SHINING LIGHT ON HIBERNATOR GENOMES: USING RADIATION TO REVEAL DNA DAMAGE AND REPAIR DYNAMICS IN ARCTIC GROUND SQUIRRELS

By

Krysta L. Yancey, B.S.

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APPROVED:

Andrej Podlutsky, Committee Chair
Kelly Drew, Committee Member
Michael Harris, Committee Member
Diane Wagner, Chair
Department of Biology and Wildlife
Leah Berman, Dean
College of Natural Science and Mathematics
Michael Castellini, *Dean of the Graduate School*

Abstract

Mammalian hibernation is characterized by dynamic changes in metabolism and body temperature; it may be sustained for up to nine months in some species. The majority of hibernation is spent in torpor, a dormant state, which is regularly interrupted by brief periods of activity referred to as interbout arousal. Upon arousal thermogenesis begins in vascularized fat and ends with whole-body shivering until the animal reaches a body temperature around 36-37 °C. Interbout arousal is usually less than a day long and is commonly thought to be necessary for maintenance and repair of tissues, in addition to the cycling and replenishment of metabolites. While physiologically extreme, torpor-arousal cycles do not drastically impact the health of hibernators. Rather, hibernators are recognized for their longevity and resistance to a variety of stresses, such as ischemia/reperfusion and the brain damage that typically follows. It remains unknown how the process of hibernation challenges genome stability and the basic molecular mechanisms of DNA repair. Therefore, this thesis begins with a review on current knowledge of genome maintenance in the context of mammalian hibernation, distinguishing it from other similar and often correlated conditions like hypothermia. Then, we present the first cellular and molecular study to be conducted on DNA damage and repair dynamics in a hibernator using the Alaskan arctic ground squirrel. Our results indicate that hibernators can avoid genome instability during torpor-arousal cycles through status-specific combinations of strategies for preventing DNA damage and efficient DNA repair, paired with anti-apoptotic environments. The unique suite of adaptations necessary to endure torpor-arousal cycles may help explain the longevity and radio-resistance that are often observed in hibernating species.

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General Introduction

A subset of mammalian species are capable of reducing their metabolism and body temperatures by entering a period of torpor. Mammals that demonstrate fluctuating internal body temperatures such as this are called "heterotherms." Heterothermy is phylogenetically diverse and present in all three main mammalian branches: monotremes, marsupials, and eutherians. Among eutherians, heterothermy is found in the orders Afrosoricida, Carnivora, Chiroptera, Erinaceomorphia, Rodentia, and Xenarthra (1). Heterothemy has even been observed in Primates – Madagascan dwarf lemurs and mouse lemurs, specifically (2, 3). Mammalian heterothermy is physiologically and behaviorally complex, varying among species, but is broadly classified as daily torpor if lasting fewer than 24 hours or sustained torpor (hibernation) if longer (4). Daily torpor differs from sustained torpor in more than duration; hibernators decrease their metabolism and body temperature more evidently (5). As indicated by the name, daily torpor is also tightly coupled with the molecular circadian clock while seasonal hibernators do not have this dependency (5). So, where daily torpor and hibernation have been thought to exist on a continuum (4), there is evidence that they may be distinct adaptations instead (5). Despite uncertainty behind the differences between daily torpor and hibernation, there is consensus that heterothermy is almost certainly an ancestral, plesiomorphic trait (6). In this thesis, the term torpor will be reserved for periods of hypothermia and hypometabolism, while hibernation will reference the whole seasonal process of torpor-arousal cycles.

Classically, heterotherms are depicted in cold environments, but they are also present in tropical and subtropical environments. The occurrence of heterothemy across this wide variety of

climates and species provides strong evidence that torpor is directly linked with food availability (1, 7, 8). In the event of food shortage it is advantageous to reduce metabolism and energy expenditure until more favorable conditions return. Hibernators are called obligate in areas where food availability is seasonally-dependent, such as cold climates, for these species will hibernate predictably on a circannual rhythm (6). Alternatively, facultative hibernators are typically found where food shortages are unpredictable or seasonally-independent; such as occurs with droughts, freezing, or other conditions that temporarily affect food availability (6). With these strategies, hibernating mammals will expend 4% to 25% of the energy needed to otherwise remain at euthermic temperatures over equivalent time, in a species-dependent manner (9).

Hibernation may be sustained for up to nine months in some species, but not all time during hibernation is spent torpid. Rather, hibernators regularly exit torpor and undergo interbout arousal (IBA) for brief periods of time before returning to torpor. Body temperature begins rising by increased brown adipose tissue metabolism, joined later by whole-animal shivering to reach normothermia. As parameters of metabolism, respiration and heart rate also increase during this time, meeting the demands for oxygen availability. Regardless of a return to metabolic activity, some species will not eat, drink, defecate, or urinate during this time while other species may engage in some activity (10). After spending a short period of time in euthermia animals re-enter torpor. In a highly coordinated manner, metabolism decreases, matched by reductions in respiration and heart rate. As the demands for oxygen diminish, so will its supply. These torpor-arousal cycles occur in most hibernators, which signifies that torpor, while an energy-saving strategy, is not sustainable for the entirety of the hibernation season and animals must

periodically increase metabolic rates and temperatures. The reason behind the necessity for IBA interruptions of torpor is thought to be for cellular maintenance and for the restoration of metabolic homeostasis (11, 12). The regulation of torpor and bouts of arousal are strongly linked to thermoregulation in the hypothalamus (13).

The goal of this thesis was to investigate genome maintenance during hibernation at a cellular and molecular level. Chapter 1 is a mini-review on what is known about DNA damage, radio-resistance, DNA repair and other related processes in hibernators. Given the surge in using hibernation-like states not only to increase the viability of organs for transplant, but also the efficacy of cancer treatments and future space travel where radiation exposure is high, it is important to establish what effects hibernation has on genome maintenance. Chapter 2 then compares radio-resistance, DNA repair dynamics, and cellular viability of peripheral blood mononuclear cells isolated from Arctic ground squirrels throughout the year. This study concludes there are temperature-independent, status-specific differences in how radiation damage is processed and ultimately expressed on a cellular level.

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Chapter 1: A Mini-Review on Genome Maintenance and Hibernation[1]

1.1 Abstract

Hibernation is characterized by dynamic fluctuations of temperature, respiration, and brain activity. The periods of dormancy are termed "torpor" and the brief periods of activity are referred to as "interbout arousal" (IBA). During torpor metabolism decreases and, for many species, body temperature rests near ambient temperature. Intermittently, thermogenesis will increase and body temperatures will briefly return to normothermia (~36-37 °C) along with most other physiological processes before dropping again. The exact utility behind IBA is unknown, though it is suggested that IBA facilitates maintenance and repair of tissue, cycling and replenishing of metabolites. Hibernators are recognized for their longevity and tolerance to a variety of physiologically stressful conditions, especially oxidative stress and cardiac arrest. However, it remains unknown how the process of hibernation challenges genome stability and the basic molecular mechanisms of DNA repair. Among other factors, fluctuations in body temperature, chromatin compaction, and antioxidant availability throughout hibernation develop a complex environment where some aspects are protective and others are potentially deleterious for DNA. It is important to establish the outcome for DNA given the surge of interest for applying hibernation-like states in medical procedures, such as organ transplant storage or cancer treatments where radiation exposure is high. This review focuses on what is known about DNA damage, radio-resistance, DNA repair and other related processes in hibernators.

[1] Yancey, K. and A. Podlutsky. A mini-review on genome maintenance and hibernation. Prepared for submission to the *Journal of Gerontology*.

1.2 Hibernation

Heterothermy is an evolutionarily ancestral characteristic for mammals, with most clades including at least one species that has demonstrated the capacity for daily torpor and/or hibernation (1, 2). Classic examples of hibernation research are found among the orders of Rodentia, Chiroptera, and Carnivores (3), but with recent observations of hibernation-like states in the Madagascar lemur (*Cheirogaleus medius*) there is a resurgence in exploring the capacity for primate hibernation (4). Across diverse environments where hibernators are found, from arctic to tropical conditions, the primary drive behind the evolution for hibernation appears to be a lack of food (3, 5, 6). With regular reductions in food availability, it is an advantageous strategy to reduce metabolism and energy expenditure until favorable conditions return. Obligate hibernators will enter hibernation annually, on a predictable schedule, and are typically found where food availability is tightly influenced by seasonality, such as winter in polar climates (1). On the other hand, facultative hibernation occurs in response to drought, freezing, or other conditions that may temporarily and unpredictably affect food availability. Overall, hibernating heterotherms will only expend between 4% and 25% of the energy that would be necessary to maintain normothermia over an equivalent time period, depending on the species (7).

The physiological changes that accompany mammalian hibernation are well characterized. Hibernation is comprised of two physiologically distinct states, that of sustained torpor and the brief bouts of arousal that interrupt it. During torpor, metabolism is severely depressed in comparison to the basal metabolic rate (BMR) observed during summer normothermia (8). For example, the average metabolism during torpor is 25% of BMR in Alaskan black bears (*Ursa americanus*) (9) and as low as 5% of BMR in rodents (7). Both a

decrease in metabolism and ambient temperature contribute to the drop in body temperature often observed during hibernation, but minimal attainable body temperatures are largely regulated in a species-specific manner (7). Black bears (*U. americanaus*), for example, can survive body temperatures no lower than ~30 °C (9), but arctic ground squirrels (*Spermophilus parryii*) hold the record for the lowest recorded mammalian body temperature at an impressive -2.9 °C (10). It should be noted that, for this arctic rodent, colder ambient temperatures in the hibernacula (e.g. -12 °C) incur proportionately higher metabolic costs to maintain their species-specific minimum body temperatures (11).

Sustained torpor is regularly interrupted by interbout arousal, characterized by a whole-body rewarming effort that brings body temperature back to ~37 °C. This sudden surge in thermogenesis begins by increasing brown adipose tissue metabolism and progresses to a whole-body effort apparent through vigorous shivering (8). Interbout arousal is energetically taxing, often accounting for the majority of energy expenditure during hibernation. For Arctic ground squirrels (*S. parryii*) in 4 °C hibernacula, up to 86% of the energy expended during hibernation is used for interbout arousal (11). As body temperature increases through metabolic activity, so do related physiological processes of heart and respiration rate (8). Overall, oxygen supply and delivery are well coordinated with tissue demands, maintaining homeostasis. While the process of interbout arousal is well characterized, the underlying utility for arousal during torpor remains elusive. One hypothesis, prompted by observations that ground squirrels spend most of their time during euthermia sleeping, suggests that sleep debt accumulates during torpor and influences the timing of arousals (12, 13). Even though further research suggests otherwise (14), the concept that arousals facilitate cellular maintenance and restoration remains (8). This idea is further

supported by the removal of potentially toxic plasma metabolites and restoration of free fatty acid and amino acid stocks during arousal (15).

Recent research continues to reveal the complex molecular mechanisms underlying the physiological changes of hibernation. For example, the phenotypic changes that are characteristic of torpor-arousal cycles may be largely attributed to genome-wide transcriptional regulation (16-21). In this review, we focus on genome maintenance during mammalian hibernation, which has received little attention in literature regardless of its importance in cellular functions and aging (22).

1.3 Hibernation Models

Research on hibernating species should be contextualized within the limitations and challenges of the field. Early hibernation research on whole animals focused on broad, non-invasive physiological measurements, such as respiratory patterns and temperature fluctuations (23). Modern biotelemetry techniques with internal and external sensors and instruments record more parameters of physiology and are especially useful in studies on free-ranging wild animals in their natural hibernacula (24). Cellular and molecular biology, in addition to streamlined "-omics" technology, further develops a picture on how hibernators tolerate such stresses associated with sustained dormancy for up to nine months of a year (8). It is a challenge, however, to experiment on very specific cell pathways *in vivo* in whole animals. The standard for studying mammalian DNA damage and repair pathways is with specialized cell-lines, but hibernation researchers do not necessarily have this convenience.

Cell-lines have been established from hibernating mammals, where the unique characteristics of hibernator-derived tissue translate to cell-culture. For example, hibernating species are resistant to the deleterious effects of hypothermia regardless of season, a characteristic that translated into cultured hamster kidney cells (25). Likewise, brain-slices from Arctic ground squirrels are tolerant to modeled ischemia regardless of season (26, 27) and so are their cultured neuronal progenitor cells (28). The appeal of establishing cell-lines is not only the ability to manipulate variables, but also to utilize assays that require plated or suspended cells.

Cell cultures may help to establish the differences between non-hibernators and hibernators, but it is unlikely they are capable of representing differences between the actively-hibernating and summer seasons. Hibernation is a complex process involving coordination among organs to elicit tissue-specific changes that make up the observed phenotype (29). When cells are removed and propagated from species capable of hibernation, they presumably lose the cellular environment and accompanying cues that would otherwise occur circannually. Even with these shortcomings, however, cell-cultures will help to minimize variables and to focus on specific pathways and mechanisms.

Early on, hibernation was singled out as an example of naturally-occurring hypometabolism and hypothermia in mammals (30). The appeal for mimicking these conditions in non-hibernating mammals, like humans, are the many proposed applications. Lethal hypoxia was survived, for example, with the use of hydrogen sulfide (H_2S) to induce reversible torpor-like hypothermia in mice by suppressing oxygen consumption (31, 32). Similarly, attenuating thermogenesis by using antagonizing adenosine A_1 receptors in the central nervous system in rats

resulted in decreased body temperatures and increased survival following asphyxial cardiac arrest (33). Recently, utilizing adenosine monophosphate increased survival after a lethal dose of radiation by putting mice into a hypometabolic state (34). It has been noted, however, that subjecting non-hibernating species and cell cultures, by extension, to hypothermia through these methods is not necessarily equivalent to the complex process of hibernation. Hibernation is an evolutionarily adaptive response that is likely triggered by multiple cues in a highly coordinated effort that is circannually regulated (29).

1.4 DNA Damage and Radio-resistance

In a series of experiments from the 1950s-60s involving whole-squirrel irradiation, hibernation was associated with protection against ionizing radiation. Specifically, when mortality was compared between groups of thirteen-lined ground squirrels (*C. tridecemlineatus*) irradiated during torpor or arousal, those irradiated during torpor and immediately aroused within 2 hours showed longer survival and a total dose reduction factor of 1.4 (35). Additional research reveals that following a dose of 12 Gy whole-squirrel irradiation, blood lymphocytes and bone marrow hematopoietic cells retained their synthetic activity and overall health when treated during torpor compared to interbout arousal groups (36). However, it should be noted that hibernation has been associated with delays in the development of lethal processes initiated by radiation exposure until arousal (37), thereby artificially minimizing the impact of exposure within an acute time-period. The exact mechanism(s) underlying radio-resistance observed during hibernation in mammalian species, if it exists, remains unknown.

Radio-resistance could be attributed to any number of processes that occur between initial exposure and the final response, such as preventing the formation of genome damage in the first place. Ionizing radiation (IR) causes DNA damage by energizing water molecules (i.e. water radiolysis), leading to the formation of highly unstable reactive oxygen species (38). It is the nature of these reactive oxygen species to achieve stability at the expense of cellular components; free radicals react with compounds and leave the targeted compound with an unpaired electron and as a free radical itself. The damaged cell components include proteins, lipids, and DNA. Antioxidants end this chain-reaction by preventing or intercepting the free radicals (39). It is suggested that radio-resistance in the bacterium *Deinococcus radiodurans* and other extremophiles is achieved by nonenzymatic antioxidant pathways that prevent oxidative modifications to proteins, allowing cells to retain function of DNA repair and other regulatory pathways following radiation exposure (40, 41). Other proposed characteristics of *D. radiodurans* that may contribute to radio-resistance are: the capacity for genome transfer between bacteria (42), multiple copies of the genome per cell (43), and genome reconstitution through DNA repair (44).

Nonenzymatic antioxidants may play a similarly beneficial role against IR damage in mammals. During hibernation, mammals increase expression and/or activity levels of plasma and tissue antioxidants, such as ascorbate. In both Arctic ground squirrel and thirteen-lined ground squirrel species, levels of plasma ascorbate are three- to fourfold higher during torpor before decreasing to euthermic levels during arousal (45). The decrease in plasma ascorbate is accompanied with an increase of ascorbate in peripheral tissues. This reallocation is hypothesized to reduce the effects of oxidative stress during reoxygenation of tissues upon

arousal, especially in brain tissues (46). The hypothesis is supported as the rate of ascorbate uptake by tissue correlates significantly with O_2 consumption (46). Similar antioxidant defenses are found in the brains of hibernating bat species, where the level of reactive species is significantly lower during arousal than torpor or active states (47). It follows that avoiding the irreversible and unrepairable damage caused by oxidative stress, such as protein carbonyls, potentially prevents further energy expenditure necessary to produce new proteins during a time when energy preservation is fundamental (48). The lack of oxidative stress markers (i.e. lipid peroxidation, protein carbonyls, etc.) in tissues during arousal in many hibernating species suggests efficient coordination between pro-oxidant and antioxidant mechanisms (47, 49, 50).

However, not all tissues in mammalian hibernators show increased antioxidant defenses or a lack of oxidative stress markers. Tissues that play a primary role in thermogenesis during interbout arousal, such as brown adipose tissue, were found to have higher indicators of oxidative stress during late arousal and euthermy than during torpor (48). While the reason behind increased oxidative stress in these tissues is unknown, it may be an anticipatory defense against the premature exhaustion of antioxidant reserves before euthermia is resumed, when the reserves may be replenished.

1.5 DNA Repair

Following DNA damage, the main two mammalian DNA repair mechanisms are base excision repair (BER) and nucleotide excision repair (NER) (51, 52). BER is responsible for repairing oxidized or alkylated DNA bases, in addition to the abasic sites created through spontaneous depurination/depyrimidation (53). These lesions are the most prevalent attacks on

the genome and highly mutagenic because they do not distort the DNA backbone sufficiently to stall DNA replication forks. Any interruption in the BER pathway can lead to stable mutations. For example, if the oxidized base 8-oxoGuanine remains undetected it results in the guanine:cytosine to thymine:adenine mutation that is the second most common mutation found in human cancers (54). On the other hand, the NER pathway repairs bulky and other DNA-distorting lesions that can hinder the transcription fork (52). Overall, repair dynamics are well established for humans and rodent models, but unknown for mammalian hibernators.

The physiological changes that accompany torpor-arousal cycles may interfere with or create unique challenges for the repair pathways. A prevalent hypothesis is that interbout arousal is necessary to interrupt lengthy torpor bouts in order to accommodate cellular maintenance (8); this likely includes DNA repair. Multiple factors that restrict repair during torpor, such as decreased temperatures and DNA accessibility, reverse upon interbout arousal. It has been proposed that, because of adapting to these challenges, hibernators might demonstrate better repair capacities than non-hibernators (55). However, it is unlikely that there are novel pathways in mammalian hibernators; rather, any differences are likely due to changes in regulation (8).

1.5.1 Temperature

According to the Arrhenius equation, as temperatures decrease, so does the rate of reactions. Therefore, it is safe to presume that low body temperatures reached by hibernators would coincide with a decrease in the rate of biochemical reactions necessary for the detection, recruitment, and performance of enzymes repairing DNA damage.

1.5.2 Genome Accessibility

Along with damage detection and recruitment of repair enzymes, cells must also orchestrate genome remodeling to facilitate the association between DNA and repair complexes. Eukaryotic DNA is wrapped around histones, which together form nucleosomes. When acetyl and phosphate groups are associated with histones, the chromatin is relaxed and DNA more accessible. This relaxation is then reversible under the regulation of compaction enzymes, such as histone deacytlases (HDACs) and dephosphorylases, leading DNA to re-associate tightly with histones and block further activity.

This remodeling is especially important for NER, where the presence of nucleosomes inhibit repair enzymes from forming and performing repair (56). Hyperacetylation of histones is associated with significantly more efficient repair synthesis (57), emphasizing the likely relationship between histone-acetylation and DNA-processing enzymes, such as those involved in repair (58).

Chromatin remodeling is less implicated in BER, where the relatively smaller repair complexes may form in the linker regions between histones (58). However, evidence that ATP-dependent chromatin remodeling complexes enable BER, and that all steps of BER are negatively impacted by increased compaction, makes the role of such complexes in BER *in vivo* still under review (59).

Specific investigations on DNA repair pathways in mammalian hibernators are lacking, but many studies have been carried out on transcriptional control during hibernation. DNA-

processing enzymes (i.e. repair enzymes, transcription factors) need access to DNA in order to function. During torpor in the thirteen-lined ground squirrel (*I. tridecemlineatus*) the genome undergoes structural modifications that block transcription by rendering DNA inaccessible. These controls are reduced during interbout arousal, when DNA activity resumes for a short time (16, 19).

Overall, the relationship between compaction levels and accessibility of DNA-processing enzymes, like those involved in repair, highlight interbout arousal as favoring proficient repair dynamics. Considering compaction alone, torpor is likely to be associated with poor repair dynamics as a result of increased transcription restriction by impeding DNA access on a global scale. The question remains then, whether or not repair of incurred DNA damage would be stalled during torpor until IBA and result in accumulating damage the longer torpor continues.

1.5.3 Activity of Repair Enzymes

Transcriptome studies have indicated upregulation of DNA repair mechanisms as a strategy employed during hibernation to offset genotoxic stress. In the brain tissues of bats, RAD50, NBS1 (nibrin), and ATM were upregulated throughout hibernation in comparison to summer levels (18). RAD50 and NBS1 are two of the three proteins necessary for the formation of the MRE11/RAD50/NBS1 (MRN) complex, which recognizes double-strand DNA (dsDNA) breaks and bridges these breaks for homologous recombination (HR) and non-homologous end joining (NHEJ). This complex also serves to activate ATM in response to DNA damage, which halts cell cycle progression (60). A robust DNA repair response would serve as an offense against damage and cell death in these essential brain tissues – where cell death is less favorable

(18). It remains to be determined if similar upregulation occurs in other tissues and heterothermic species during hibernation.

The MRN complex also serves to maintain telomeres, where NBS1 may be necessary for telomere elongation by telomerase (60). Mammals, such as the Djungarian hamster (*Phodopus sungorus*), are reported to experience increases in relative telomere length (RTL) over 180 days of daily torpor, leaving them with longer telomeres (61). However, the hibernating edible dormouse (Glis glis) experiences decreases in RTL over the hibernation season, influenced by the rate of arousal (62). As a strategy to counteract the deleterious effects of arousal on RTL, the edible dormouse undergoes telomere elongation over active summer periods (62, 63). The contradictory effects of daily torpor and hibernation on RTL could be an effect of minimal obtained body temperature. By maintaining a relatively moderate torpor body temperature, daily heterotherms are able to avoid the oxidative stress and telomere damage associated with arousals from near ambient temperature that hibernators experience (62).

1.6 Programmable Cell Death

Following DNA damage, if attempts of repair fail then programmable cell death (PCD) pathways activate (64). There are three main types of PCD: apoptosis, autophagy, and necrosis. While recognized as a strategy to prevent the propagation of damaged cells, apoptosis is tightly regulated to prevent immature or inappropriate levels of cell death. If the regulation of apoptosis is dysfunctional, either too much or not enough, it can lead to many pathologies, such as autoimmune diseases (65) and neurodegenerative diseases (66). Therefore, there are anti-apoptotic mechanisms in place to delay or halt cell death (67). Many separate pathways and

cellular signaling cascades lead to apoptosis and each is susceptible to intervention by anti-apoptotic mechanisms. The balance between pro- and anti-apoptotic pathways ultimately decides cell fate (67). Cancer is a prime example of instances where increased expression of anti-apoptotic proteins (e.g. Bcl-2) or dysfunctional pro-apoptotic proteins (e.g. Bax) can lead to resistance against apoptosis and result in an overpopulation of cells (68).

There is evidence that hibernators possess adaptive responses against PCD that would be stimulated by stresses of hibernation. Global stressors of hibernation include heat and cold shock, osmotic stress, oxidative stress, hypoxia, starvation (autophagy), and metabolic stress (67, 8). Overexpression of common anti-apoptotic genes (i.e. ROS scavengers and heat shock proteins (HSP)) will typically serve to delay or prevent apoptosis in response to such global stresses (67). Hibernators have been shown to employ these common anti-apoptotic strategies. Specifically, in addition to upregulated antioxidant defenses and other ROS scavengers during hibernation, HSP70 increases during torpor entrance and exit (69), while a member of the HSP70 family, GRP75, concentration is higher in the muscle tissues of hibernating squirrels than summer-active squirrels (70). Anti-apoptotic Bcl-2 family members are upregulated in white adipose tissue in thirteen-lined ground squirrels to increase cell survival during torpor-arousal cycles (71). In hibernator-derived cell-cultures, less cell death was observed in response to hypothermia and glucose-deprivation (28, 25). The anti-apoptotic mechanisms and pathways are not established in these models yet, but there is strong evidence for mitochondrial morphology and metabolism to play a role (25). Ultimately, hibernators pose as a source for novel information on anti-apoptotic processes (67).

1.7 Cell-cycle Progression

During hibernation, many energetically taxing processes are downregulated; the ATP-demanding process of cellular division among them. As most tissues in adult animals are composed of terminally differentiated cells, it will be through examining somatic tissues that maintain proliferative capacity (e.g. stem and progenitor cells) that the profile of cell-cycle progression between torpor and arousal phases of hibernation will be understood. In this section, the relationship between cell-cycle regulation and DNA damage responses are explored in the context of mammalian hibernation.

Dividing cells progress through four stages of a cell-cycle, consisting of the mitotic-phase (M) and an interphase of G_1, S, and G_2. Cells may escape the cell cycle during G_1 to a reversible, quiescent stage (G_0) whereupon receiving extrinsic signals will reinitiate the cell-cycle (72). Most somatic tissue is terminally differentiated in G_0, but some tissues will maintain proliferative capacity (e.g. stem cells). Cell-cycle progression is tightly regulated by the activity of cyclin dependent kinases (Cdk) and their association with cyclins, proteins whose levels fluctuate in a stage-specific manner. Consequently, each stage of the cell-cycle may be identified by the presence of stage-specific Cdk-cyclin complexes (e.g. Cyclin-E—Cdk2 forms during S-phase) (73). Checkpoints occur during transitions between stages of the cell-cycle to ensure controlled division without mistakes. Cells can exit the cell-cycle during these checkpoints in response to extrinsic and/or intrinsic stressors, such as DNA damage (74). Triggering a checkpoint and arresting the cell-cycle is generally beneficial, as it allows cells to repair DNA damage or commit to PCD if necessary (67). Cell-cycle arrest occurs through Cdk inhibitors (CKI), either by competing for space at cyclin binding sites (INK4 family) or inhibiting the complex's

catalytic site (Cip/Kip family). Additionally, Cdk-cyclin kinase activity can be regulated by Cdc25 phosphatases (72). Following DNA damage by UV or IR, stress-response kinases ATR and ATM phosphorylate checkpoint kinases (Chks 1 and 2), which in turn phosphorylate Cdc25s and promote their degradation (75, 76). In high enough quantity, CKI will promote a strong signal of cell-cycle arrest characteristic of senescence – a phase of irreversible cell-cycle arrest in cases where apoptosis may not be favorable, but the risk of propagating genetic errors is still eliminated (77).

Early investigations into gastrointestinal radio-resistance of hibernating rodents were the first to indicate a cell-cycle stall at the G_1-phase during torpor (55). Modern cell-sorting techniques for constructing a cell-cycle profile, such as fluorescence-activated cell sorting, remain difficult when working with fresh animal tissue as they first require digestion into individual cells. However, techniques using gen-, transcript-, and proteomics on various tissues continue to support that torpor is characterized by cell-cycle arrest in G_1. Expression patterns of cyclin and Cdk proteins in liver tissue, paired with data on regulation pathways effecting the formation and activity of the resulting Cdk-cyclin complexes, support arrest in G_1 during early torpor and continued suppression at the G_1/S transition throughout torpor (78). Similarly, in the transcriptome of bone marrow, cyclin expression supports hematopoiesis suppression during hibernation (79). These studies both strongly indicate the mechanism of arrest at the G_1/S transition through upregulation and expression of CKI, specifically $p15^{INK4b}$ and $p21^{CIP1}$ (79, 78). As a promoter for p21 transcription, patterns of p53 expression and activity throughout hibernation support these findings (80). In addition to regulating cell cycle progression, other key effects and functions of p21 are DNA repair, modulation of apoptosis, and the induction of

senescence (81). While overexpression of p15 and p21 are typically characteristic of senescent phenotypes, it appears that during torpor cellular quiescence is occurring instead; for cycle progression continues upon arousal (78). The roles of Chks 1 and 2 in traditional cell-cycle arrest pathways were unclear, as they are upregulated during entrance into torpor and early arousal, but did not correlate with any changes in the phosphorylation status of Cdc25 (78).

Patterns of stem cell activity and tissue renovation may be extrapolated from these molecular data by observations that wound-recovery during torpor is minimal, perhaps non-existent. There are no indicators of post-operative tissue healing of skin grafts during torpor. Instead, healing proceeds when animals enter arousal (82). Even the proliferation of homologous and heterologous tumor grafts into submucosal cheek tissue of hamsters was halted during torpor, resuming upon arousal (83, 84). According to more recent research, body temperatures maintained during torpor may play a large role in tissue renovation, where lower body temperatures correlate with decreased wound-healing processes. For example, big brown bats (*Eptesicus fuscus*) that maintain body temperatures near ambient temperature during torpor demonstrate delayed wound-healing of wing biopsies, but no change in the rate of healing once healing is resumed (85). In this study, the onset of wound-healing correlated with an increase of ambient temperature from 10 °C to 16 °C (85). Similarly, American black bears (*U. americanus*), which maintain torpor above 30 °C, were able to fully heal skin biopsy wounds in the same time-frame and stages as the non-hibernating controls over 2-3 months (86). Therefore, more pronounced decreases of body temperatures during torpor are accompanied with a shift in cell cycle progression that delays proliferation until returning to warmer body temperatures.

1.8 Applications and Intellectual Merit

Given the common ancestry of hibernating and non-hibernating mammals, the ability to hibernate in response to specific triggers is likely due to differential regulation of genes (8, 87). This fact comes with the hope that, through manipulating genetic expression on non-hibernating species, we might be able to apply aspects of hibernation to cases of hypothermia, cardiac arrest, and cell death (33). These topics have been reviewed previously in detail, such as organ transplant technology (88). Even applying hibernation-like states for space travel is being revisited, given demonstrations of radio-resistance (89).

Hibernators are also applicable to aging research, as many hibernators deviate from aging theory by outliving their predicted lifespans, based on body mass alone. Indeed, hibernating bats outlive closely related non-hibernating bats, even when other factors are accounted for (90). Arctic ground squirrels on the north slope of Alaska, with body masses comparable to rats, have been observed living for over 10 years (Cory Williams (University of Alaska Fairbanks) and Jeff R. Werner (University of British Columbia), personal communications). Given the role of genome maintenance in longevity and aging theory, it is worth researching in hibernating mammals as well. For example, telomere dynamics in the edible dormouse have demonstrated the remarkable capacity for these rodents to elongate their telomeres on the "off" season, through telomerase activity in somatic tissue (61, 63).

It has been proposed that inducing hibernation-like states would be means for better cancer treatment efficacy (89). However, before patients are put into hibernation-like states, it is crucial to understand the effects of hibernation on the cell-cycle. During torpor, tissues capable

of proliferation temporarily exit the cell-cycle and rest in quiescence until the next arousal episode. Tumors grafted into the cheek pouches of hibernating hamsters also show halted cell-cycles (83, 84). This is both beneficial, in that the cancer growth is slowed, and problematic as most cancer treatments are effective against rapidly dividing cells. Moreover, cancer stem cells, which often reside in a quiescent state, would therefore still be difficult to treat (91).

Research on genomic stability and DNA repair dynamics during hibernation will contribute to concepts of evolutionary history and longevity research. Chromatin compaction is recognized as a cellular strategy for protection against DNA damage and maintaining genomic integrity (92). It is theorized that selection in favor of condensed genomes was evolutionarily critical in single cell life forms, as their repair mechanisms were rudimentary (92). However, the strategic packaging of DNA through histone wrapping is not unique to ancestral organisms and has been largely retained. For example, yeast spores, have approximately 10-fold more condensed DNA than surrounding somatic cells (93). Among all the benefits, however, there exists a need for repair enzymes to access damage – something compaction may interfere with (94). Literature remains inconclusive on whether mammalian species have adapted the capacity to locally manipulate chromatin to access damaged areas (95, 96). Hibernating species, like the Arctic ground squirrel, experience both chromatin compaction and genomic stress where efficient repair during compaction is theorized to occur. This makes squirrels a good candidate for studying the dynamics of DNA compaction and genome stability. Already the transcriptome of the hypothalamus in thirteen-lined ground squirrels has been sequenced to show that molecular adaptations for torpor include upregulated genomic protective measures, specifically proteins involved in detecting and responding to double-strand breaks (e.g. *RAD50*, *NBN*, and

ATM) (18). Skeletal muscle transcriptomics highlights similar mechanisms utilized during torpor, though through increased expression of HS90B, MAP4, and NF2L1 (17).

1.9 Conclusion

Among mammalian species, hibernators are recognized for their longevity and tolerance to a spectrum of physiologically stressful conditions, such as ischemia/reperfusion and hypothermia. There is also rudimentary evidence that squirrels will survive lethal doses of ionizing radiation more so if they are undergoing torpor. As radio-resistance is an unlikely selective pressure in these animals, the evolutionary adaptive responses to the stresses of hibernation likely have secondary effects on longevity and radio-resistance. At the surface, adaptations for torpor (decreased body temperature, increased chromatin compaction, and reduction in metabolism) appear only beneficial, but these conditions also pose to create an environment that makes the target DNA damage slow to detect and repair. Sustained torpor is interrupted with brief periods of IBA, where body temperatures rise along with the activities of DNA-metabolizing enzymes. This makes IBA more equipped to deal with genome maintenance than torpor, a realization in line with current theories that present IBA as a necessary break from torpor to facilitate cellular maintenance and metabolite turn-over.

Most hibernation-based research has been, in the past, centralized around mechanisms of metabolism and resistance against muscular atrophy and ischemia/reperfusion. We have, however, highlighted the need to additionally understand genome maintenance throughout hibernation. It will be important to establish the effects that hibernation has on DNA damage,

repair, and cellular viability within the context of suggested applications of hibernation-like states.

1.10 References

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Chapter 2: Changes in DNA Damage and Repair Dynamics during Hibernation in the Arctic Ground Squirrel, *Spermophilus parryii*[2]

2.1 Abstract

Lifespan correlates with body mass in most mammals, but many hibernators deviate from this trend by outliving their predicted lifespans. One of the hallmarks of aging is the accumulation of genetic errors on a cellular level, which generally correlates with the accumulation of DNA damage, slowing of DNA repair rates, decreased cellular viability and accumulation of senescent cells. We examined whether or not hibernation inflicts DNA damage throughout torpor-arousal cycles and how hibernation affects subsequent DNA repair in an obligate hibernating species, arctic ground squirrel (AGS). Results show that peripheral blood mononuclear cells isolated from hibernating AGS accumulated less genomic damage than those from summer-active animals. When exposed to DNA damaging X-ray irradiation *in vitro*, significantly fewer lesions were introduced in cells from hibernating animals than from summer animals, demonstrating resistance to DNA damage during hibernation. Additionally, cells from hibernating animals repair introduced lesions faster than cells from summer AGS. Hibernating AGS appear to tolerate genomic stress by first resisting DNA damage and then by repairing damage quickly, avoiding its detrimental outcomes. This genomic upkeep may help keep the genome intact during stresses of hibernation, with the benefit of delaying aging.

[2] Yancey, K. L., Rice, S., Frare, C., Bhowmick, S., Brines, D., Peterson, A., Drew, K. L., and A. Podlutsky. Changes in DNA Damage and Repair Dynamics during Hibernation in the Arctic Ground Squirrel, *Spermophilus parryii*. Prepared for submission to the *Journal of Gerontology*.

2.2 Introduction

Over the past century, the world-average lifespan of humans increased from a mere 30 years to 70 years (1). Such change is attributed to advancements in medicine and sanitation (1). Lifespan is not expected to increase as rapidly in the future, however, because the primary biological factors that influence the rate of aging are not clearly understood and further improvements of sanitation will likely not dramatically impact the average lifespan in developed countries (1, 2). In mammals, longevity quotients (LQ) reveal an empirical relationship between lifespan and body mass, but many hibernators deviate from this trend by outliving their predicted lifespans, up to 9.8 times longer than the LQ values predicted for bats (3, 4). The organisms studied here, Alaskan arctic ground squirrels (*Spermophilus parryii*; AGS), are obligatory hibernators that live up to 10 years in the wild, which is twice as long as non-hibernating rodents of similar size (personal correspondence; J. R. Werner, University of British Columbia; C. Williams, University of Alaska Fairbanks). One of the most popular aging hypotheses proposes that the ability to repair cellular damage declines over time, resulting in an accumulation of errors and damage that in turn affect metabolic processes and result in genetic instability and deteriorating health associated with aging (1). Therefore, preventing DNA damage and/or repairing DNA damage well will help sustain life and its quality (5). Evidence suggests that the underlying mechanisms used to tolerate physiological stresses associated with hibernation may also have the benefit of delaying aging (2, 6).

AGS are ideal model organisms for studying defense mechanisms against the stress of hibernation, because of their low body temperatures during a prolonged period of hibernation – up to nine months. For example, they can tolerate an extreme reduction in body temperature,

down to -2.9 °C; the lowest mammalian body temperature recorded (7). AGS then overcome hypothermia and return to euthermia (36-37 °C) every 1 to 3 weeks throughout their hibernation season in a process termed interbout arousal (IBA) (8). During IBA they demonstrate tolerance to physiological conditions detrimental to human health, especially hypoxia and transitioning from aerobic hypometabolism to normal metabolic rates (9).

As AGS progress from the torpor state of inactivity to an active state of high oxygen consumption, large amounts of mitochondria-produced reactive oxygen species (ROS) are theorized to cause oxidative stress (9, 10). When carbonyl proteins and lipid peroxidation were measured as indicators of oxidative stress in brown adipose tissue, they were present in high amounts during IBA (9). However, no one has yet reported whether this rush of ROS is accompanied with increased DNA damage, or how hibernation affects DNA repair mechanisms.

We hypothesized that DNA damage resistance, coupled with improved DNA repair dynamics, increase cellular stress tolerance in hibernating species such as AGS compared to non-hibernating species, the rat. These two features together may afford AGS the benefit of longevity compared to other non-hibernating rodents of similar size.

In the present study we report the results from experiments that tested: (1) whether the transition from torpor to IBA is accompanied with increased DNA damage; (2) whether hibernation provides endogenous defense to genomic lesions from ROS intermediates introduced through *in vitro* ionizing radiation or ultraviolet C (UV-C) light exposure; (3) whether hibernation increases the proficiency of base excision or nucleotide excision DNA repair

mechanisms in comparison to summer months; and (4) whether the viability of isolated white blood cells 24 hours following exposure to ionizing radiation decreases equally among statuses in AGS.

2.3 Materials and Methods

2.3.1 Animal Husbandry and Tissue Collection

All animal-related procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC #491809 and #467983), where blood collection is covered under the opportunistic sampling clause. Wild adult and juvenile arctic ground squirrels (AGS; *Spermophilus parryii*) of both sexes were trapped along the Dalton Highway in the northern foothills of the Brooks Range, Alaska in July, approximately 40 miles south of Toolik Field Station of the University of Alaska, Fairbanks (68°38'N, 149°38'W; elevation 809 m) and transported to Fairbanks, AK animal housing facility. The AGS were housed in environmental chambers set to 18 °C with a natural light cycle of summer conditions, referred to hereon as "summer AGS." After an adjustment period of 2 weeks, blood was collected representing summer conditions. In mid-August, chamber settings were changed to 2 °C and a 4:20 hour light:dark (L:D) light cycle to induce hibernation. Hibernation is characterized by sustained torpor interrupted by regular bouts of arousal and it naturally occurs up to 8 months; we collected AGS blood from late-September to late-January. After 3-7 days of torpor, during which respiratory rates reached < 6 respirations/min and body temperature (T_b) rested near ambient temperature (T_b = 2-4 °C), we collected blood samples representative of torpid AGS. Late-arousal AGS blood collections consisted of AGS emerging from torpor and

achieving T_b > 34 °C. Rats (*Sprague Dawley*; Simonsen Labs, Gilroy, CA) were used for a rodent comparison and kept in chambers set to 20 °C with a 12:12 L:D light cycle.

AGS and rat blood samples were collected opportunistically, either collecting with a heparin syringe during euthanasia or through cannulas in living animals. Torpor and arousal AGS stages were verified by rectal T_b. Euthanasia was conducted for research unrelated to this project. For details on surgical procedure, see previous publication (11). The nature of opportunistic collection limited each animal to providing one specimen, representative of its current status. The final result was 15 independent rat, 8 torpid AGS, 11 interbout arousal AGS, and 9 summer specimens.

2.3.2 Assays for DNA Damage and Repair Responses

We used two sources of genomic stress, X-ray or UV-C irradiation, to introduce DNA damage. To determine the percent of DNA damage in the peripheral blood mononuclear cells (PBMCs) we used single cell gel electrophoresis, known as the "comet assay." The comet assay, as described in further detail elsewhere (12), is a well-established and standard technique for measuring the percentage of DNA damage in PBMCs and many other cell types (13). Within an hour of collection, fresh blood samples were mixed with low-melting point agarose (1.5% in phosphate-buffered saline (PBS)) for a final concentration of 0.75% agarose and applied onto microscope slides. X-ray treatments (Cabinet X-ray System; R650; Faxitron X-ray Co.) were administered at a rate of 1.22 Gray/min for total doses of 0.65, 1.21, 1.86, 3, 6, or 9 Gray (Gy), verified through the use of a dosimeter (Cobia Sense; Southern Scientific Ltd.). UV-C treatments (UV Stratalinker 1800; Stratagene) were administered at doses of 5, 10, 20, or 40 J/m^2, detected

by an internal dosimeter. In order to measure the rate at which the PBMCs removed DNA lesions induced through gamma radiation, cells were allotted up to 90 mins following X-ray or up to 6 hours following UV-C to undergo DNA repair within a high-glucose Dulbecco's Modified Eagle Medium (DMEM) growth media (Life Technologies, Carlsbad, CA) inside of a CO_2 incubator (ThermoScientific, Waltham, MA) at 37 °C. To prepare the slides for electrophoresis, they were rinsed with phosphate-buffered saline (PBS) and transferred to a lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton-X 100, 10% DMSO, pH 10) for 1 hour at 4 °C in the dark. Afterwards, slides were transferred to fresh neutralization buffer twice (5 min, 0.4 Tris, pH 7.5) and placed into an alkaline electrophoresis solution (200 mM NaOH, 1 mM Na_2EDTA, pH > 13) at 4 °C for 20 minutes to equilibrate pH and unwind DNA. Following electrophoresis (20 min, 25 V, 300 mA) in the alkaline solution, slides were again rinsed twice in neutralization buffer, then fixed in 100% ethanol for 5 minutes and air dried before stored in a dark box. After staining the slides for DNA with SYBR-Green (Sigma-Aldrich, St. Louis, MO), images of nuclei were captured at x20 magnification with an EVOX FL fluorescent microscope (ThermoFisher Scientific) using a GFP filter. For each dose within treatment, a minimum of 100 nucleoids were analyzed – if possible – for the percent of damaged DNA and tail moment with software by one technician (Comet-IV ©; Instem, Bury St. Edmunds, UK).

2.3.3 Assay for Cellular Viability

In order to assess cellular viability following treatment, ratios of live to dead white blood cells were obtained with the Muse ® Count and Viability Assay Kit (Millipore, St. Louis, MO). In order to isolate white blood cells, fresh whole blood samples were combined with ChemCruz © Red Blood Cell Lysis buffer per manufacturer's instructions (10 min, 1X dilution made in DI

H_2O) before a final suspension in RPMI growth media (Life Technologies, Carlsbad, CA). Alternatively, white blood cells were isolated with Ficoll © density gradient centrifugation per manufacturer's instructions. The cells were then divided into control and treatment groups. X-ray was administered to treatment groups at a rate of 1.22 Gy/min for a total dose of 12 Gy (Cabinet X-ray System; Faxitron X-ray Co.). Following incubation of 24 hours, cells were centrifuged and washed with PBS twice (5 min, 350 rcf) before staining (5 min) at a final working cell concentration between $1x10^5$ and $1x10^6$. During torpor in AGS, circulating white blood cell counts decrease, which was compensated for with larger blood samples, when possible.

2.3.4 Statistical Analysis

As comet assays are known for producing right-skewed data, given the zero-inflated nature of the assay (14), we log-transformed data where assumptions of normality were not met. Assumptions were not met (strong right-skew) for UV dose- and repair-response data, so the log transformation was used before means were derived for each experiment. We report the means ± standard deviation for each status, transformed where necessary. For comparison of background DNA damage (covariate) among AGS statuses (fixed effect), we used a one-way ANOVA with a pairwise Tukey's HSD test to correct for multiple comparisons. Most regressions were linear except for the X-ray dose-responses which were best described with the equation $y = (y_{max}*x)/(k+x)$, where y_{max} = the maximum DNA damage achieved and k = the dose at which the DNA damage is half of y_{max}. Using the R package 'drc' for analysis of dose-response data, y_{max} and k values were found with the 'drm' function, in which Micheaelis-Menton starting values were used to find the best fit, and compared by one-way ANOVA. We report the calculated y_{max} and k values ± standard error for each status. In regressions that included time (continuous

effect), such as DNA repair, we used a one-way ANCOVA with a pairwise Tukey's HSD test. In this study p-values less than 0.05 are considered significant. Intraspecies single comparisons were performed between summer AGS and rats using a Student's t-test. Dose responses to X-ray and UV-C irradiation were analyzed for differences using regression analysis with R, version 3.5.1 (15). Repair is represented in proportion to the initial damage incurred upon exposure (i.e. percent change). Repair responses are represented in terms of half-life of DNA damage, τ_{50} ("tau fifty"), and calculated from the equation $\tau_{50} = (c\text{-}50)/(\text{-}b)$, where the slope ($b$) and y-intercept ($c$) are determined from regression lines. In other words, τ_{50} is the measure of time until half of the initial DNA damage is repaired.

2.4 Results

2.4.1 DNA Damage during Hibernation

The percent of background DNA damage detected in PBMCs from torpid, aroused, and summer AGS was not significantly different ($p = 0.14$; $F(2, 23) = 2.13$) (Figure 2.1). IBA was associated with the lowest amount of DNA damage (2.26 ± 1.09%), followed by summer (4.41 ± 2.83%) and torpor squirrels (4.73 ± 4.57%) (Figure 2.1). Overall, these findings show that the background level of DNA damage does not change considerably between the time-points sampled.

While there was no statistically significant difference in background DNA damage among AGS statuses, there was a significant difference between summer AGS and rats ($p = 0.033$). We measured 2.50 ± 0.77% DNA damage in rats, nearly half of that observed in summer AGS (Figure 2.1).

2.4.2 Dose-Response to Ionizing Radiation

In order to study dose response to ionizing radiation, PBMCs were treated with various doses of X-ray irradiation at a rate of 1.22 Gy/min. The main effect of AGS status ($p < 0.001$; $F(2, 137) = 10.84$) is statistically significant, explaining the differences between dose-response curves (Figure 2.2). Curve constants for each status may be found in Table 2.1. Pairwise comparisons were only significantly different between summer and IBA AGS groups, where IBA samples incurred an average of 8.75% less damage than summer ($p = 0.002$). Summer samples scored more damage than torpor until 5 Gy, when torpor samples start incurring more damage than summer samples. Summer AGS and rat curves follow a similar pattern, as the rat and torpor curves are nearly identical.

2.4.3 Repair-Response to Ionizing Radiation

In order to ascertain repair dynamics following a 3 Gy dose of X-ray exposure, the amount of DNA damage was monitored up to 90 minutes post-irradiation. Our results show that, following the introduction of DNA damage from ionizing radiation, status has a significant effect on overall damage present over time ($p < 0.001$; $F(2, 62) = 12.37$) (Figure 2.3). With only one replication of torpor X-ray repair experiments, where the internal replicates averaged one tenth that of the other statuses, interpretation of torpor data requires caution. We have plotted torpor repair response data for visual comparison and preliminary statistics, but otherwise it is insufficient for any conclusive remarks. Data with small sample sizes, such as torpor in this instance, is extremely prone to the influence of outliers as seen in Figure 2.3 where torpor's 60 minute data point does not follow the trend of repair. In light of this, there was significantly less

DNA damage over time in IBA squirrels than summer ($p < 0.001$) or torpor ($p < 0.01$). However, interestingly, there is no significant difference between summer and torpor ($p = 0.62$). Likewise, there was no significant difference between summer AGS and rats ($p = 0.68$).

The DNA damage half-life, τ_{50} , was then calculated for each animal and averaged (Figure 2.4). The calculated τ_{50} for IBA was 80.81 ± 51.29 minutes; significantly lower than summer ($p = 0.035$; $t = 3.11$), which averaged 177.49 ± 42.62 minutes. The τ_{50} of DNA damage from cells isolated during torpor was 185.83 minutes and not significantly different from any other status – likely because of the small sample size. For rats, τ_{50} was 136.92 ± 79.99 minutes and not significantly different from summer AGS ($p = 0.33$). Overall, the DNA repair dynamics of AGS following exposure to ionizing radiation are largely similar to those of the non-hibernating rats, except for during IBA when they are almost twice as fast.

2.4.4 Viability Following Ionizing Radiation

To assess if tolerance to genotoxic stress is a factor in long-term cell survival and observed radio-resistance during hibernation, we measured the viability of PBMCs 24 hours after ionizing radiation during different statuses (Figure 2.5; Table 2.2). Among the AGS samples, only PBMC isolated from IBA squirrels showed a significant decrease in cellular viability after exposure ($p = 0.03$; $t = 3.75$; $df = 3.20$), decreasing 24.35 ± 10.84% compared to the control of non-irradiated cells. Interestingly, the viability of cells isolated from torpid and summer AGS did not decrease within the observed timeframe in response to radiation. The rat comparison responded to treatment by significantly decreasing viability to 66.52 ± 14.71% of the control ($p < 0.001$; $t = 6.65$; $df = 8.67$). Therefore, among AGS statuses, IBA is the only status associated

with decreased viability following ionizing radiation and summer AGS are less likely to enter programmable cell death than rats.

All viability assays scored greater than 1600 cells per assay, except for torpor which only scored an average of 100 cells per assay (Figure 2.6; Table 2.2). The difference in sample size was primarily due to the sequestering of lymphocytes in organs during torpor and therefore a decrease in the concentration of circulating lymphocytes available for sampling. The correlation coefficient between viability and the number of events scored was -0.23, implying a slight inverse relationship between the two variables. As the number of events scored increases the calculated viability slightly decreases, but not significantly enough to reject the null hypothesis that the slope is zero ($p = 0.13$). Consequently, the number of events scored did not influence the variation observed in viability either as a main effect or as an interaction with other variables ($p > 0.05$), and so was dropped from the model. In the end, viability was best explained by the main effects of animal status and treatment group.

2.4.5 Dose-Response to UV-C Light

Bulky adducts and other helix-distorting alterations to DNA may be created *in vitro* through UV-C electromagnetic irradiation (16). To study the effect of UV-C exposure on cells isolated from hibernating animals, we subjected PBMCs to different doses of UV-C light: 5, 10, 20, or 40 J/m^2. Unlike X-ray damage, which creates abasic sites required for the comet assay immediately after exposure, UV-C damage creates dimers and other forms of DNA damage that are not observable with the comet assay immediately. As cell repair machinery begins to excise nucleotides in an attempt to approach and remove the UV-C DNA damage, abasic sites are

created which in turn are detectable with the comet assay. In this study, we sampled 1 hour after UV-C exposure to quantify percent DNA damage. A byproduct of this process is large variation among internal replicates, as cells are observed in the beginning, middle, or end of this process.

In this study, a one-way ANCOVA reveals both main effects of AGS status ($p = 0.0032$; $F(2, 79) = 6.183$) and dose ($p < 0.001$; $F(1, 79) = 110.71$) to be statistically significant variables that impact the resulting DNA damage (Figure 2.7). There was no significant interaction between the two variables. Tukey's HSD pairwise comparisons show that, on average, cells from torpor are 7.07% more sensitive to UV-C light compared to IBA ($p = 0.0065$) and 7.17% more than summer ($p = 0.0040$). IBA and summer groups did not exhibit a difference ($p = 0.99$). Similarly, there is no average difference between summer AGS and rat groups ($p = 0.75$).

2.4.6 Repair-Response Following a Single UV-C Light Exposure

Unlike ionizing radiation, the damage introduced to DNA from UV-C radiation does not express itself as lesions/breaks in DNA immediately upon exposure. DNA damage is only detectable with the comet assay as DNA repair machinery excises bases in order to replace them, leaving single-stranded DNA which breaks in the high pH environment of the electrophoresis chamber. In our assay, the time in which the DNA damage was the highest following exposure of 20 J/m^2 was chosen as the "expression time" and all consecutive time-points were standardized against it to calculate the half-life of DNA damage (τ_{50}).

Our results show that status did not have a significant effect on overall damage present over time ($p = 0.097$; $F = 2.47$; $df = 2$) (Figure 2.8). The largest overall difference was between

IBA and torpor ($p = 0.11$). Within the IBA status, some animals repaired the damage, while others did not, which may explain why there is large variation. Likewise, there was no significant difference between summer AGS and rats ($p = 0.53$).

The DNA damage half-life, τ_{50}, was then calculated for each animal (Figure 2.9). Status was a significant predictor of average τ_{50} ($p = 0.032$; $F(2,7) = 5.84$). There was a significant difference between the average τ_{50} of IBA and torpor ($p = 0.027$; $t = 3.41$). The τ_{50} from cells isolated during torpor was 10.96 ± 3.68 hours and no repair occurred during IBA, represented by a negative value -0.28 ± 6.37 hrs. Summer had the fastest τ_{50} at 4.91 ± 1.34 hours, but no significantly different than that of rats, 13.39 ± 8.87 hours ($p = 0.15$; $t = 1.89$). This is inconclusive, however, as IBA data are conflicting. One animal repaired, while the other did not. There is large variation in DNA damage present over time following UV-C light, given the nature of how nucleotide excision repair occurs. Therefore, derived means and standard deviations are not necessarily appropriate representations of repair dynamics.

2.5 Discussion

In this study, we report for the first time that AGSs, obligate hibernators, demonstrate seasonal fluctuations in DNA-damage resistance and subsequent DNA-repair dynamics to either ionizing radiation or UV-C light. Likewise, cellular viability following ionizing radiation is also status-specific. Compared to summer controls, animals undergoing IBA are both less susceptible to ionizing radiation and demonstrate almost twice as fast repair dynamics. Counterintuitively, however, only samples derived from IBA animals decreased in viability after X-ray treatments while the other statuses did not. Following UV-C light exposure, samples derived from torpid

animals incurred the most damage, and responded with relatively less efficient repair than other statuses. The repair results following UV-C light exposure in IBA samples are inconclusive, with some cells repairing others demonstrating no repair. These results support status-specific mechanisms of genomic protection during hibernation and may also help maintain the long-term genomic stability necessary for longevity.

2.5.1 Genomic Stress of Hibernation

During the transition from torpor to IBA, oxygen consumption and body temperature increase dramatically, as do indicators of oxidative stress (9). The observed oxidative stress is notably tissue-specific, tending to correlate with metabolically active organs during the transition, such as brown adipose tissue, and absent otherwise (e.g. liver and brain) (9). PBMCs are often used as a measure of genomic stress on an organism scale, because they circulate and are representative of general conditions throughout the body (13). We measured the background DNA damage present in PBMCs and our results showed no significant difference in the amount between torpid, IBA, and summer AGS (Figure 2.1). Metabolism during torpor in AGS is reduced to approximately 1% of normal basal metabolic rate (8) and so, theoretically, by lowering their metabolism during torpor, hibernating mammals would also reduce the rate of ROS generation and subsequent genomic damage (17). However, our results might indicate that DNA damage is accumulated during the torpor phase and then repaired upon IBA, given that low temperatures are known to delay repair (18), which could explain the low amount of DNA damage observed during IBA.

2.5.2 DNA-Damage Resistance during Hibernation

The ROS intermediates produced from metabolism that cause DNA lesions may be created *in vitro* through X-ray irradiation (19). In our study, samples obtained from IBA squirrels demonstrated the most radio-resistance against ionizing radiation, averaging 8.75% less damage than summer controls ($p = 0.002$) (Figure 2.2). However, samples isolated from torpid squirrels responded similar to summer samples. Given that ionizing radiation damages DNA through ROS-intermediates, the same products of metabolism, the inherent resistance to ROS-induced DNA damage during IBA may additionally account for the reduced amount of background damage observed (Figure 2.1).

Several mechanisms of DNA-damage resistance could be at work in AGS, which undergo physiological and molecular changes in preparation for hibernation (20). One such mechanism is chromatin compaction. For example, in the thirteen-lined ground squirrel DNA transcription rates are globally restricted throughout hibernation by chromatin compaction (21-26). When acetyl and phosphate groups are associated with histones, DNA is exposed and accessible. Under the regulation of compaction enzymes like histone deacetylase (HDAC), however, these groups are removed and DNA associates tightly with histones, blocking further activity (27). During torpor in thirteen-lined ground squirrels, analysis of HDAC expression and activity was upregulated in brown adipose tissue and muscle, and was accompanied with less acetylated and phosphorylated histones during hibernation (21, 24). Furthermore, compaction can directly reduce the amount of DNA damage introduced by ionizing radiation, theoretically by "molecularly crowding" the DNA and reducing the density of water molecules that serve as intermediates between radiation and ROS production (28). Therefore, genomes of hibernating

species undergo structural changes to reduce rates of transcription and such compaction also reduces susceptibility to DNA damage. Here we have shown that hibernating AGS undergoing IBA demonstrate more DNA-damage resistance than summer AGS. While the specific mechanisms behind this resistance to genomic stress remains elusive, upregulated compaction in order to restrict transcription is a likely mechanism behind resistance to genomic stress.

When challenged with UV-C light, cells isolated from torpid squirrels incurred significantly more DNA damage than summer and IBA statuses (Figure 2.7).
IBA samples responded similarly to summer controls. The large variation within experiments and between experiments makes any conclusions difficult. UV-C damage and repair dynamics are challenging to elucidate with the comet assay and other measures of damage may be better suited for future studies, such as antibody-based ELISA, Slot-Blot (or Dot-Blot) or immunofluorescence. This is, however, the first study to use UV-C light exposure in a study on hibernating species' stress tolerance.

2.5.3 Seasonal Variation in DNA-Repair Dynamics

It has been proposed that DNA repair is difficult for torpid organisms because of the temperature restrictions on enzyme activity (2). Others have noted, however, the benefits of delayed DNA repair as would be expected to occur in hibernators as they arouse from torpor (18, 20). We found that in addition to the observed radio-resistance to ionizing radiation, PBMCs from AGS undergoing IBA demonstrated almost twice as fast repair dynamics than those from summer and torpid AGS (Figure 2.4). Although torpor experienced the most damage following UV-C light it exhibited repair, albeit slowly, where IBA samples did not repair within the time

allotted (Figure 2.8). Rodents are notoriously bad at nucleotide excision repair, the pathway initiated by UV-C damage (29). To our knowledge this is the first documentation of status-specific DNA-repair dynamics in a hibernator. It remains to be determined if this variation is unique to the AGS species or ubiquitous across mammalian hibernators.

While specific investigations into DNA repair pathways of hibernating mammals are lacking, many studies have been carried out on transcriptional control during hibernation. DNA metabolizing enzymes such as DNA-repair enzymes, transcription factors, and RNA polymerases all require access to DNA. During hibernation the genome undergoes structural modifications, such as compaction, that block transcription by rendering DNA inaccessible. These transcriptional controls relax slightly during IBA, but do not return to levels observed during summer months (21, 24). Similar transcriptome studies in the cerebral cortex reveal DNA-repair pathways are upregulated throughout the hibernation season, contributing to their stress tolerance and cell survival during ischemia/reperfusion (22, 23). Specifically, transcript levels for components involved in DNA damage detection and the recruitment of DNA repair mediators (i.e. RAD50, nibrin (NBN), and ATM) are upregulated in the hypothalamus during both torpor and IBA (23). Looking into the transcriptome of thirteen-lined ground squirrels, some DNA-repair associated proteins are upregulated during hibernation (22). Overall, these findings align with the hypothesis that IBA serves to accommodate cellular maintenance, such as DNA repair.

That AGS are both resistant to ionizing radiation genome stress and also show efficient repair during IBA raises further questions about possible underlying mechanisms. Compaction is

unlikely to be the sole mechanism of protection, because it may reduce DNA repair capacity (28). For example, in non-hibernating species such as humans, spermatocytes use chromatin compaction to terminate transcription and maintain the information in their high-value genomes (30). However, all DNA repair mechanisms are inhibited as well, leaving spermatocytes vulnerable to stable mutations or even sterility (30). Here we show that cells isolated from IBA AGS demonstrate resistance to genomic damage while retaining the specificity of detection and efficiency of base excision repair. The differential regulation of nucleotide excision repair pathways during hibernation are inconclusive and deserve more attention in the future.

2.5.4 Viability following Ionizing Radiation

After a high dose of ionizing radiation, cells may initiate programmable cell death pathways when DNA repair is not possible or preferable. Following radiation exposure, it is not only the amount of DNA damage that influences cell fate, but also the cell response to genomic stress. Some cells resort to programmable cell death quickly following DNA damage, while others demonstrate great resistance to apoptosis after incurring the same amount of DNA damage (19). Dividing cells are particularly sensitive to the effects of ionizing radiation and so lethal doses often result in gastrointestinal distress and hematopoietic syndrome (i.e. decrease in number of white blood cells) (31). While previous studies showed hibernating animals demonstrate radio-resistance, their assays were based on animal mortality and therefore lacked the resolution necessary to discern underlying mechanisms (32). Therefore, we sought to find the relationship between ionizing radiation exposure and cellular viability in PBMCs isolated from AGS in different statuses.

In this study, only cells isolated from squirrels undergoing IBA decreased cellular viability following a dose of 12 Gy (Figure 2.5). Remarkably, neither torpor nor summer groups responded with a statistically significant decrease in viability compared to non-irradiated controls (Figure 2.5). Many hibernators that can tolerate a variety of cellular stresses during hibernation season regain susceptibility in the summer (33). However, some stress tolerances are seasonally-independent and even translate to cell culture, such as hypothermia tolerance observed in hamster derived kidney cells (34). AGS have shown seasonally-independent resistance to ischemia/reperfusion in the brain, the only mammalian hibernator species to do this (35). This characteristic even translated into cell culture of neuronal stem cell progenitors (36). Our data suggest that tolerance to genotoxic stress is also seasonally-independent in AGS, but has yet to be demonstrated in cell-culture.

In conclusion, PBMCs isolated from IBA are associated with radio-resistance to ionizing radiation and faster repair dynamics, but are more likely to resort to cell death. On the contrary, cells from torpid squirrels incur the same amount of DNA damage and repair the damage no faster than those from summer squirrels, but are more resistant to cell death than IBA. Strong anti-apoptotic mechanisms have been implicated in hibernation, as cell death and renewal is an energy-demanding process (for review see Yancey and Podlutsky, unpublished). Alternatively, it is possible to speculate that during IBA cells are more sensitive to the damaging effects of ROS by-products and more easily commit to apoptosis. Such a phenomenon might be an example of adaptive responses to cellular stress that ultimately prevent damaged cells from cancerous transformation and as such, would be viewed as protective. Given that many stress tolerances in

hibernating animals are tissue-specific and differentially regulated, it is not unreasonable to expect multiple strategies to arise.

2.5.5 Future Directions

This study demonstrates that hibernation status affects the susceptibility to DNA damage and the ability to repair damage, independent of the effects of temperature. However, we do not rule out the role of temperature on the dynamics of DNA damaging and repair processes, as temperature fluctuations are a distinct characteristic of mammalian hibernation and the accompanying physiology. Likewise, temperature has a role in cell survival; hypothermia postpones DNA repair of double-strand breaks and increases cell survival (18). Even more relevant is the higher survival post-irradiation found in mice treated with adenosine monophosphate, which puts them into a hypometabolic and hypothermic state near 30 °C (37). Our results suggest that the hibernation phenotype and its adaptive responses also are beneficial, independent of the effects of temperature. Further research will reveal to what level the hibernation phenotype and hypothermia are redundant or have synergistic effects. During sustained torpor in AGS, regardless of the profoundly low body temperature, some processes continue functioning such as those for lipid metabolism (38). It is unknown how the DNA damage and repair dynamics in torpid squirrels at hypothermic body conditions operate in comparison to those reported here.

2.5.6 Conclusion

Alaskan AGS, obligate hibernators, live longer than non-hibernating rodents of comparable body mass. Although underlying mechanisms used to tolerate stresses associated with hibernation may delay aging, these mechanisms remain unknown. Genome stability plays

an important role in both stress tolerance and longevity, by preventing DNA damage and/or repairing DNA damage efficiently. Our results show that during hibernation, IBA AGS are more resistant to ROS-mediated genome lesions and repair these lesions quicker than during the summer months. Overall, AGS demonstrate endogenous methods for genomic upkeep that may have the unintended benefit of delaying aging. Future research into the molecular mechanisms underlying DNA maintenance and repair in hibernating mammals may reveal further associations between genome stability and aging in mammals.

2.6 Figures

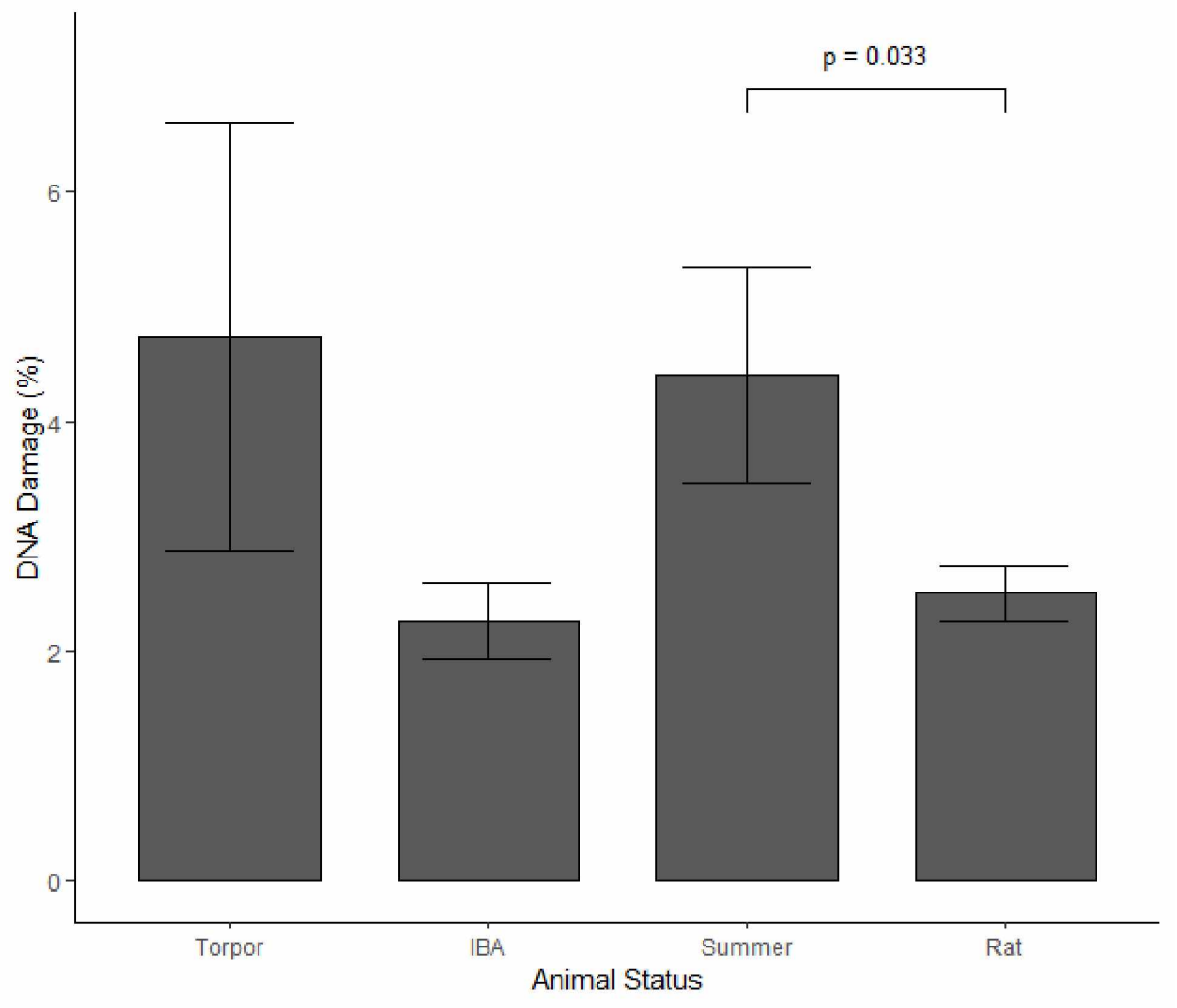

Figure 2.1. Background DNA damage among different statuses: torpor (n = 6), interbout arousal (IBA; n = 11), summer (n = 9), and rat (n = 11). Means ± standard error bars.

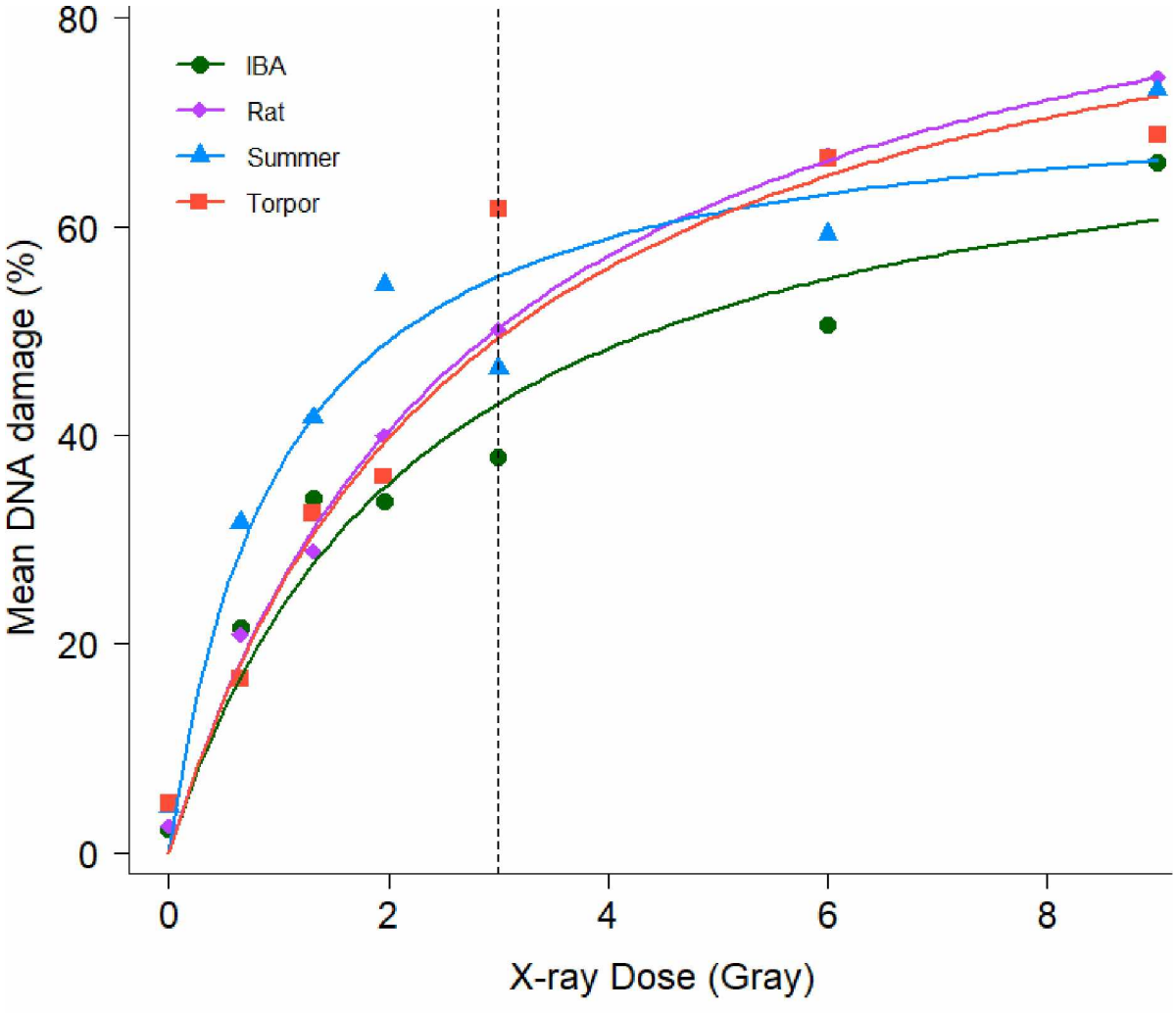

Figure 2.2. Hibernating and non-hibernating species' dose response to X-ray radiation. Means with non-linear trend lines defined by the equation $y = (y_{max}*x)/(k+x)$, where y_{max} = the maximum DNA damage achieved and k = the dose at which the DNA damage is half of y_{max}. For calculated constants see Table 2.1. For doses less than 3 Gy: IBA (n = 4), Rat (n = 7), Summer (n = 4), and Torpor (n = 5). For doses equal to and greater than 3 Gy: IBA (n = 6), Rat (n = 4), Summer (n = 5), and Torpor (n = 1). Sample sizes change where indicated by dashed line.

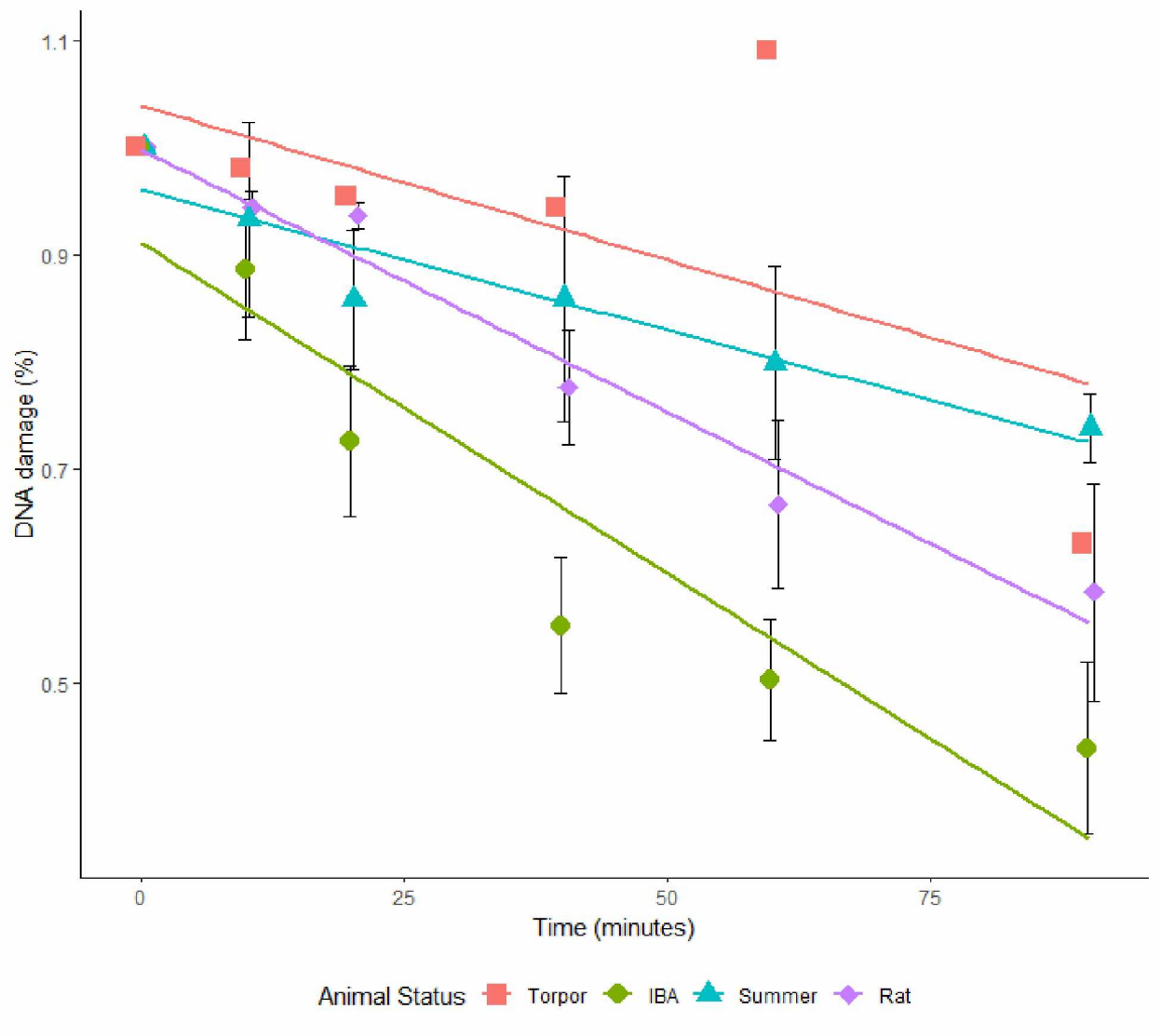

Figure 2.3. Repair response following 3Gy X-ray dose. Interbout arousal (IBA; $n = 6$), torpor ($n = 1$), summer ($n = 4$), and rat ($n = 6$). Means ± standard error bars. Points have been dodged slightly to better view error bars, but have no effect on trend-lines. Torpor data only plotted for visual comparison, as low internal replicate number makes it susceptible to the influence of outliers.

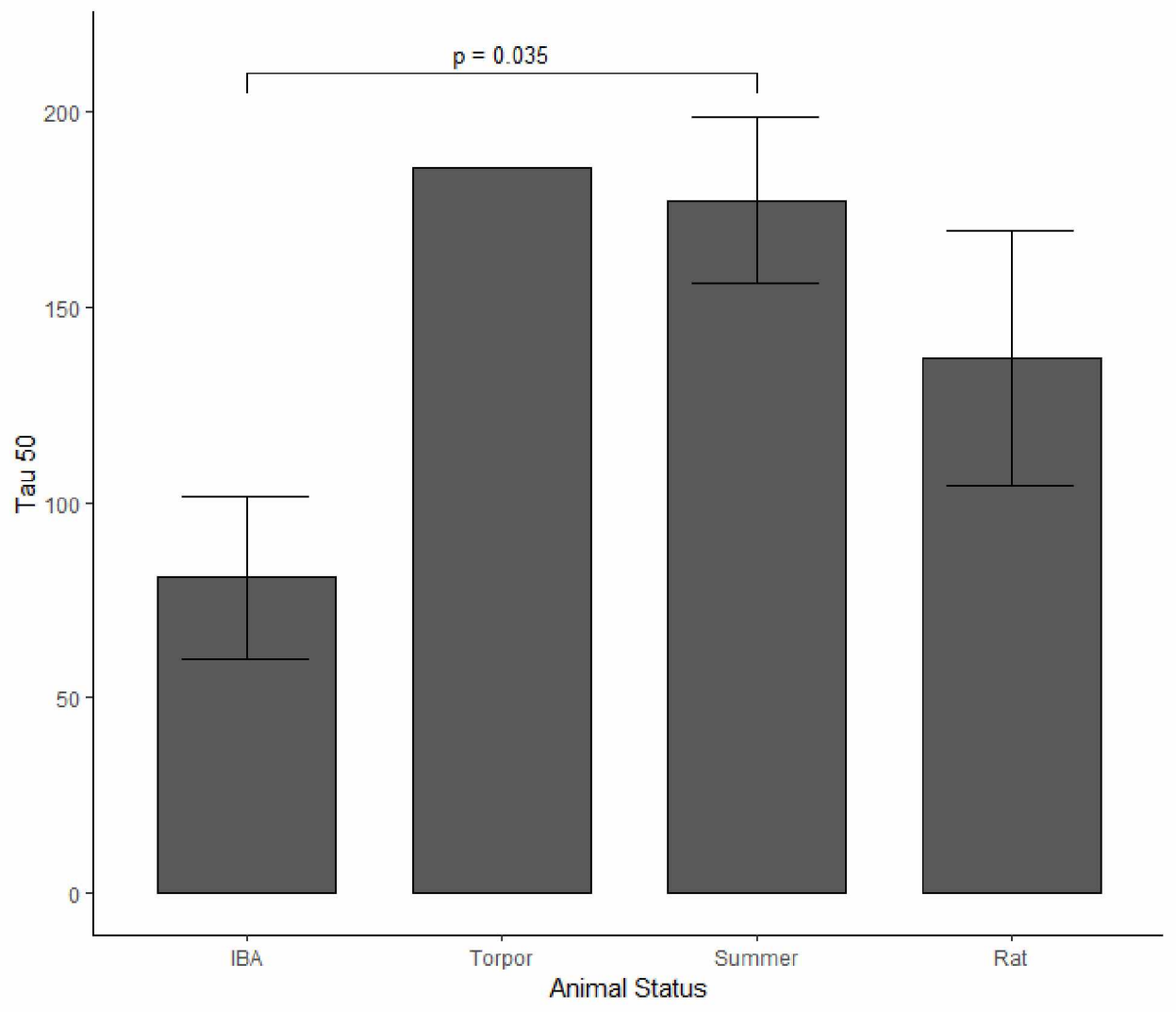

Figure 2.4. DNA damage half-life, Tau 50, for each status: interbout arousal (IBA; $n = 6$), torpor ($n = 1$), summer ($n = 4$), and rat ($n = 6$). Means ± standard error bars.

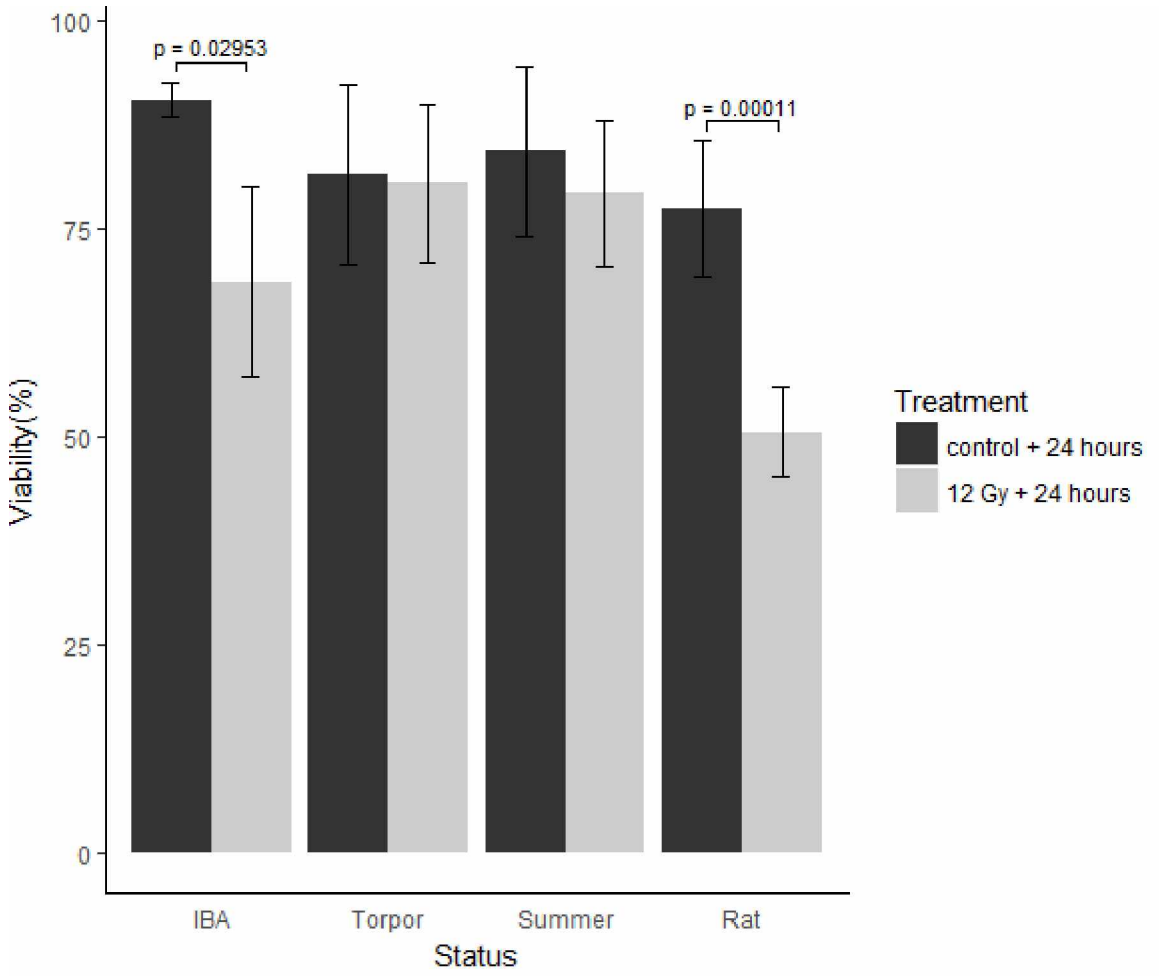

Figure 2.5. Percent viability of isolated PBMCs following radiation treatments. Means ± standard deviation. IBA squirrels (interbout arousal; $n = 4$); torpor squirrels ($n = 8$); summer squirrels ($n = 4$); and rats ($n = 6$).

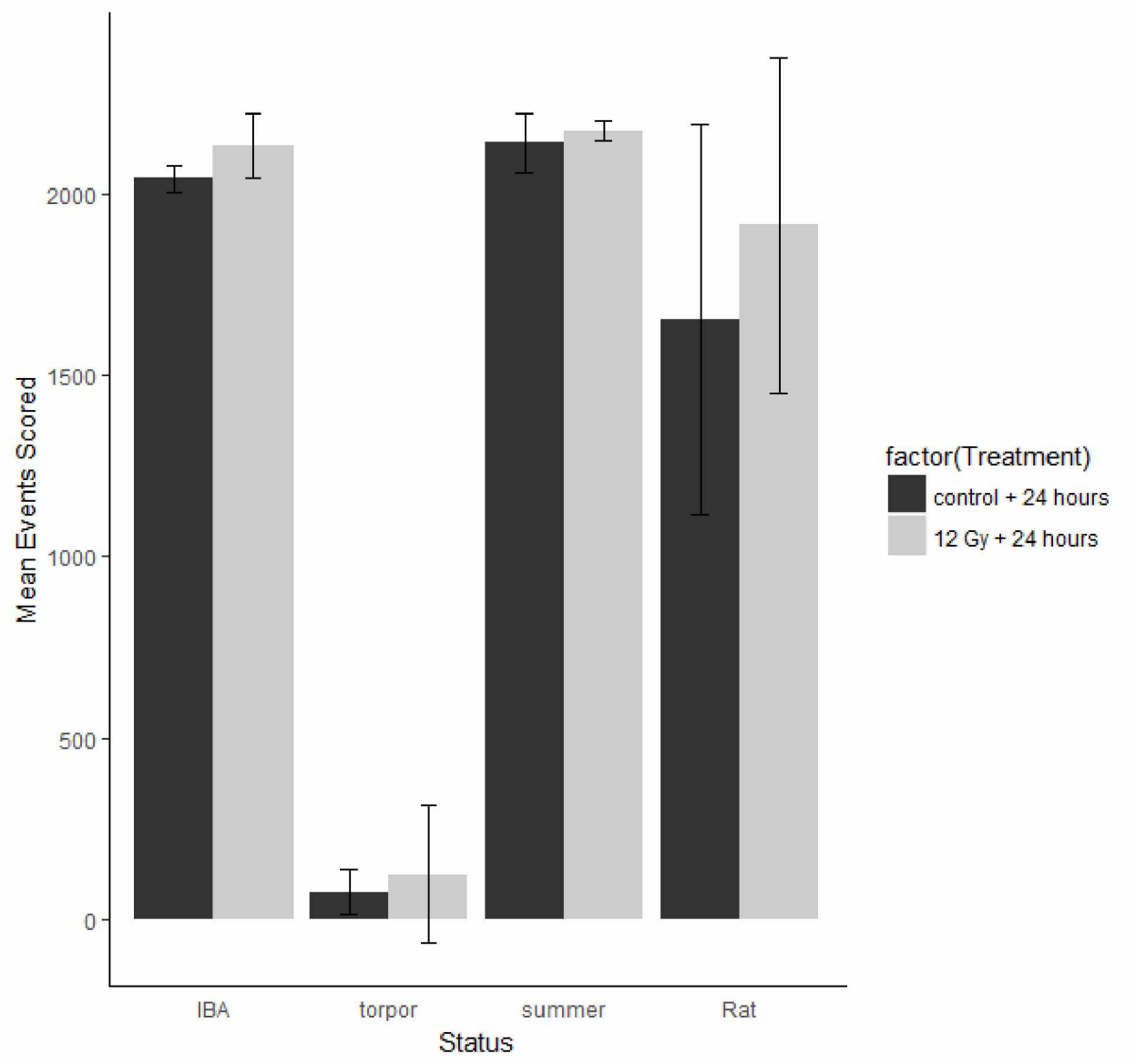

Figure 2.6. Mean number of events scored during the viability assay ± standard deviation. IBA squirrels (interbout arousal; $n = 4$); torpor squirrels ($n = 8$); summer squirrels ($n = 4$); and rats ($n = 6$).

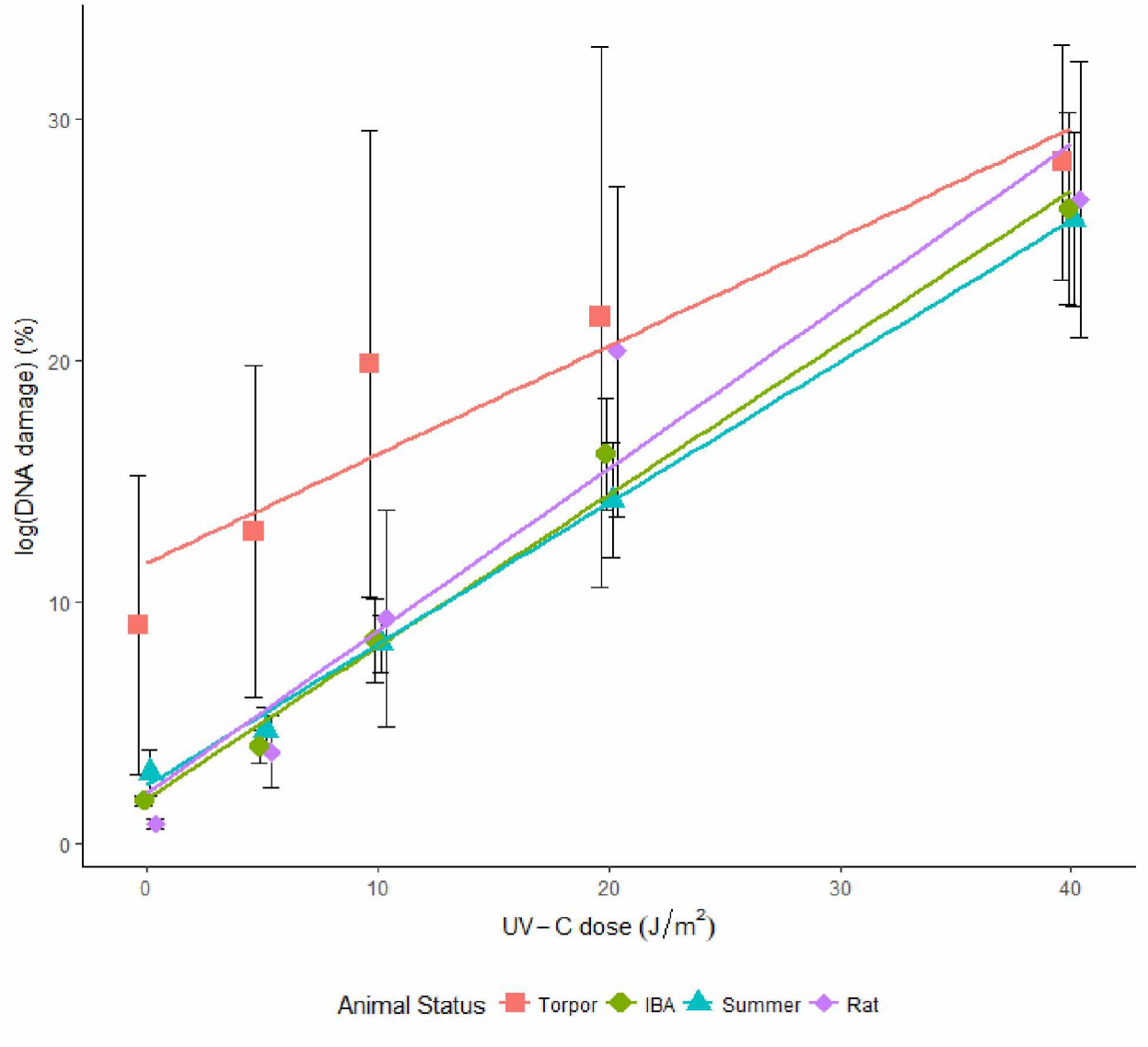

Figure 2.7. Dose response to UV irradiation. Torpor (n = 3), interbout arousal (IBA; n = 3); summer (n = 4/5); and rat (n = 4). Means ± standard error of log transformed data with trend-lines. Points have been dodged slightly to better view error bars, but have no effect on trend-lines.

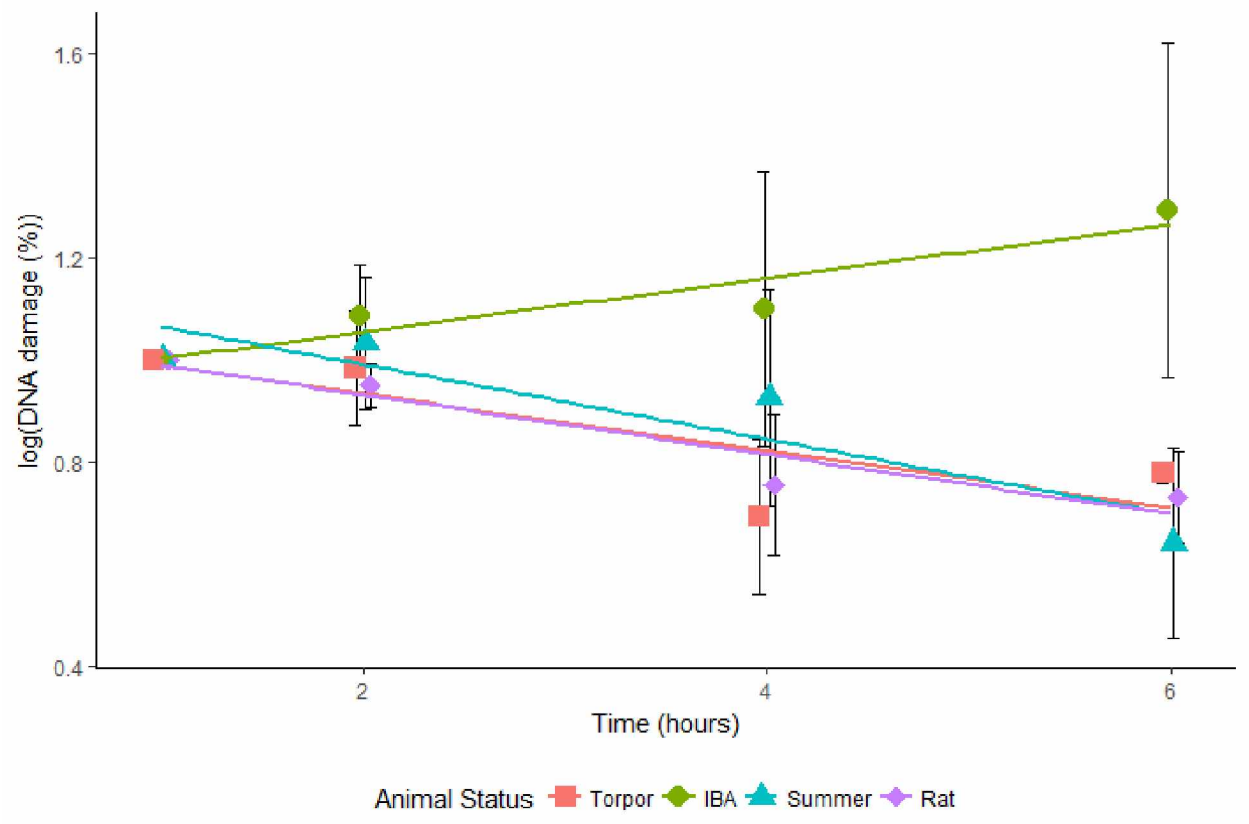

Figure 2.8. Repair response following 20 J/m^2 of UV-C radiation: torpor (n = 3), interbout arousal (IBA; n = 3); summer (n = 4/5); and rat (n = 4). Means ± standard error with trend-lines. Points have been dodged slightly to better view error bars, but have no effect on trend-lines.

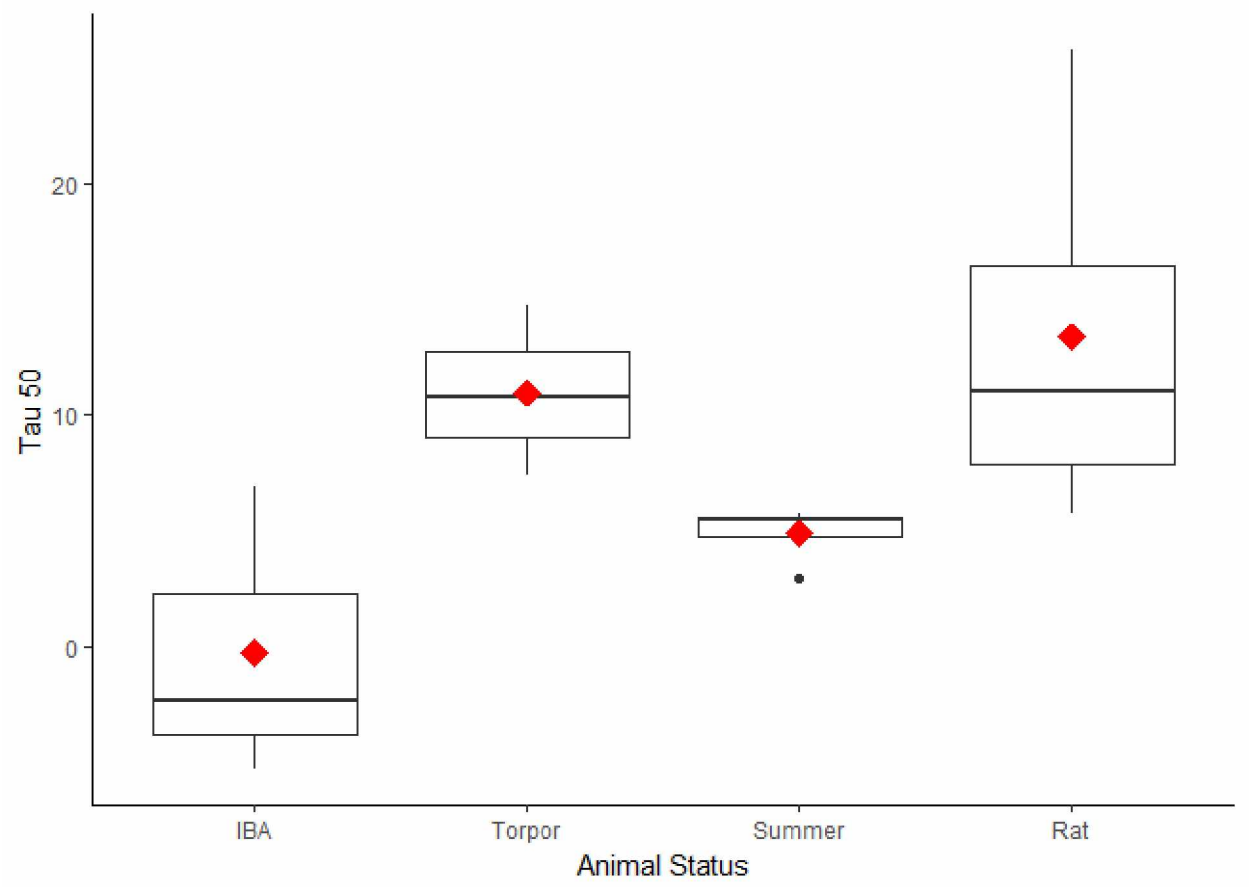

Figure 2.9. Boxplot of calculated tau-50 for each status, following exposure to 20 J/m^2 UV-C light: torpor (n = 3), interbout arousal (IBA; n = 3); summer (n = 4/5); and rat (n = 4). Thick bar = median, box = 25-75% percentiles, whiskers = 95% confidence intervals. Means are represented by red diamonds.

2.7 Tables

Table 2.1. Constants for the equation $y = (y_{max}*x)/(k+x)$, calculated from the mean DNA damage (y) present in response to X-ray dose (x), where y_{max} = the maximum DNA damage achieved and k = the dose at which the DNA damage is half of y_{max}. Means ± standard error (SE). aAGS (interbout arousal); tAGS (torpor); sAGS (summer).

Status	y_{max} ± SE	k ± SE
aAGS	76.16 ± 4.83	2.31 ± 0.41
tAGS	94.86 ± 8.69	2.77 ± 0.48
sAGS	73.74 ± 3.87	1.01 ± 0.20
Rat	97.59 ± 4.90	2.82 ± 0.32

Table 2.2. PBMC viability per treatment group, with relative treatment percentage to control. aAGS (interbout arousal); tAGS (torpor); sAGS (summer); *n* = number of samples.

Status	*n*	Dose	Viability ± SD	Relative Viability	Events ± SD	T value	Df	P value
aAGS	4	0	90.61 ± 2.09	100%	2040 ± 37.99	3.7546	3.1994	0.0295
		12	68.72 ± 11.47	75.65% ± 10.84%	2133 ± 89.577			
tAGS	8	0	81.63 ± 10.85	100%	75.5 ± 60.04	0.21866	13.734	0.830
		12	80.52 ± 9.43	100.42% ± 18.52%	124.75 ± 187.95			
sAGS	4	0	84.38 ± 10.27	100%	2140 ± 81.91	0.74125	5.8707	0.487
		12	79.35 ± 8.85	94.61% ± 11.20%	2173 ± 27.20			
Rat	6	0	77.51 ± 8.26	100%	1654 ± 538.57	6.6548	8.6647	0.000111
		12	50.62 ± 5.46	66.52% ± 14.71%	1913 ± 462.59			

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General Conclusion

This review and study has helped to highlight the unique challenges of genome maintenance faced by mammalian hibernators. In Chapter 1 I review what is known and speculated about DNA damage and repair processes that would take place in an organism that dramatically experiences fluctuations in both metabolism and body temperature. Aspects of hibernation that are traditionally considered protective may actually restrict proper DNA damage responses to the short periods of euthermia that regularly interrupt sustained torpor. However, given the longevity of mammalian hibernators and the absence of cancer, hibernators have the adaptive responses to properly coordinate genome maintenance and avoid the deleterious pathologies that typically stem from genome instability (1). Such an orchestration would not be surprising, as mammalian rodents already demonstrate it with their metabolism. For example, both the demand for and the delivery of oxygen in tissues are coordinated to the extent that ischemia and reperfusion are avoided in the majority of their tissues (2). This is possible, in part, by adaptive differences in the function and morphology of their mitochondria (3). Specifically, a dispersed mitochondrial network and higher respiratory and glycolysis capacity, though the significance of these differences remains unknown (3).

Then, in Chapter 2, using ionizing radiation and UV-C light exposure I was able to establish the trends of DNA damage and repair across different AGS statuses, independent from the effects of temperature. The results from this study were two-fold. First, interbout arousal was revealed to be accompanied with increased radio-resistance, and the rate of DNA repair was nearly twice as fast as all other groups sampled. This may help explain why interbout arousal

was associated with the least amount of background DNA damage, regardless of metabolic rates being similar to those of summer squirrels. Second, the sensitivity of AGS PBMCs to genomic stress was studied with a cellular viability assay and shown to be status-specific. Interbout arousal was the only status that behaved similarly to the non-hibernating rodents, by decreasing cell viability following radiation. Overall, these investigations highlight AGS as a prime organism to research genome maintenance as their sensitivity to DNA damage and capacity for repair vary seasonally.

Recommendations for future research include sampling throughout hibernation with higher specificity to the critical periods of transition between statuses. For example, sampling a few days into torpor and a couple weeks after sustained torpor will help establish the rate at which DNA damage accumulates. Additionally, sampling during the rush of metabolism that accompanies the onset of interbout arousal, when the much-needed reservoir of antioxidants might be exhausted, is a transition where DNA could be vulnerable. Another aspect of genome maintenance and repair that would be relatively simplistic to monitor is the transcriptome and proteome as it will indicate the potential for responses to DNA damage. Interbout arousal may serve as period of preparation against anticipated stresses that will accompany entrance and exit of torpor. Evidence for this comes from the production and turn-over of enzymatic and non-enzymatic antioxidants that appear to be essential in preventing ischemia/reperfusion upon exiting torpor. Therefore, monitoring the activity and presence of DNA damage detecting, repair enzyme recruiting, DNA polymerases, and other DNA metabolizing proteins and their precursors will reveal if genome maintenance is a higher priority throughout hibernation than it is during the summer months.

In addition to these research directions, this study has highlighted the need to include temperature as a variable in future studies. In this study, whole blood was isolated from AGS and all experiments were conducted at room temperature, regardless of the body temperature of the squirrel upon sampling. Therefore, samples taken from torpid squirrels that are maintaining body temperatures of 4°C should be kept appropriately cold and processed accordingly to determine if observed radio-resistance during torpor stems from hypothermia rather than an adaptation specific to the hibernation phenotype. Upon establishing kidney cell-culture from AGS tissue, it may also be determined if the hypothermia tolerance expressed by hamster-derived kidney cells occurs in this mammalian hibernator as well (3).

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